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COLLAGEN BINDING INTEGRINS AND CANCER TESTIS ANTIGENS IN PROSTATE CANCER AND MELANOMA

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To my family

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Department of Biochemistry, MediCity Research Laboratory, University of Turku, Finland
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ABSTRACT

Prostate cancer is the second most common cancer in men worldwide. The incidence of melanoma, in turn, is increasing faster than any other cancer incidences. In Finland, more than 5000 prostate cancer and 1200 new melanoma cases are diagnosed each year. One approach to further understand the cellular processes involved in prostate cancer and melanoma is to gain better knowledge about alterations in gene expression and their potential impact on the progression of the diseases. This thesis is focused on expression studies in two gene families; integrins and cancer testis antigens (CT antigens), in human prostate adenocarcinoma and advanced human melanoma. Integrins are heterodimeric transmembrane receptors which regulate many important cellular processes such as cell proliferation, migration and survival. CT antigens are frequently expressed in different types of cancers, but are only expressed in testis in healthy individuals. CT antigens are also highly immunogenic proteins. Due to the properties mentioned above, integrins and CT antigens can function as target molecules for the development of cancer diagnostics and drugs.

One of the main purposes of this thesis was to study the expression of the four collagen binding integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, $\alpha 11\beta 1$ and the cancer testis antigen 16 (CT16) in cancer cell lines and human tissues of prostate cancer and metastatic melanoma. Additional aims included studies on the biological role of CT16 and the abundance of CT16 in sera of advanced melanoma patients. The prognostic and diagnostic significance of CT16 and the collagen binding integrins were also evaluated. Expression studies on collagen binding integrins and the CT antigen CT16 in melanoma and prostate cancer were limited and the biological role of CT16 was unknown.

In this thesis, the expression levels of $\alpha 2\beta 1$ and $\alpha 11\beta 1$ were found to be significantly altered in prostate cancer tissues. Integrin $\alpha 2\beta 1$ decreased gradually during disease progression while $\alpha 11$ was elevated in prostate carcinoma compared to healthy tissues. In advanced melanoma, enhanced levels of $\alpha 2$ were associated with a significant shorter overall survival in advanced melanoma. In this thesis, CT16 was identified as a frequently expressed melanoma CT antigen with an anti-apoptotic function.

To conclude, this thesis presents $\alpha 2\beta 1$ and CT16, as potential and promising biomarkers for advanced melanoma. This thesis reports also the first functional study of CT16.

Keywords: Collagen binding integrins, $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, $\alpha 11\beta 1$, Cancer Testis antigens, CT16, melanoma, prostate cancer, expression

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TIIVISTELMÄ

Eturauhassyöpä on toiseksi yleisin syöpä miehillä maailmanlaajuisesti. Melanoomatapausten lukumäärä lisääntyy muita syöpiä nopeammin. Suomessa todetaan yli 5000 uutta eturauhassyöpä- ja 1200 melanoomatapausta vuosittain. Tutkimalla geenien ilmenemismuutoksia ja muutosten mahdollisia vaikutuksia syövän etenemiseen voidaan ymmärtää solujen toimintaa. Tämän väitöskirjan tavoitteena oli selvittää integriinien ja syöpä/kives antigeenien (CT antigeenit, eng. Cancer Testis antigens) ilmentymistasoja eturauhassyövässä ja melanoomassa. Integriinit ovat heterodimeerisiä solukalvoreseptoreita, jotka säätelevät tärkeitä soluprosesseja, kuten solujen kasvua, erilaistumista ja migraatiota. CT antigeenit ovat immunogeenisiä proteiineja, jotka ilmentyvät eri syöpätyypeissä. Terveissä kudoksissa niiden ilmentyminen rajoittuu kiveksiin. Edellä mainittujen ominaisuuksien perusteella integriinit ja CT-antigeenit soveltuvat mahdollisesti kohdemolekyyleiksi syövän diagnostiikkaan ja lääkekehitykseen.

Kollageenireseptori-integriinien $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$ ja $\alpha 11\beta 1$ ja syöpä/kives antigeenin CT16 ilmentymisestä melanoomassa ja eturauhassyövässä on saatavilla vain vähän tutkimustietoa. Tässä tutkimuksessa selvitettiin sekä kollageenireseptori-integriinien että CT16 antigeenin ilmentymistä ihmisen syöpäsolulinjoissa, ihmisen eturauhassyöpäkudoksissa ja metastaattisissa melanoomakudoksissa. Tavoitteena oli myös tutkia CT16 antigeenin biologista tehtävää ja sen ilmentymistasoa melanoomapotilaiden seerumeissa. Lisäksi arvioitiin CT16 antigeenin ja kollageeni-integriinien merkitys diagnostiikassa ja melanooman etenemisennusteessa.

Tutkimuksessa $\alpha 2\beta 1$ ja $\alpha 11\beta 1$ integriinien ilmentymisen todettiin muuttuvan merkittävästi eturauhassyöpäkudoksissa. Integriini $\alpha 2\beta 1$ -ilmentymistaso väheni taudin etenemisen aikana ja integriini $\alpha 11\beta 1$ määrä puolestaan havaittiin kohonneen eturauhassyövässä normaaliin kudokseen verrattuna. Kohonneet $\alpha 2$ -ilmentymistasot metastaattisessa melanoomassa ennustivat potilaan lyhyempää elinaikaa syöpädiagnoosin jälkeen. CT16 antigeenin todettiin ilmentyvän runsaasti metastaattisessa melanoomassa ja CT16 antigeenillä havaittiin olevan antiapoptoottinen vaikutus soluihin. Tutkimuksessa esitellään $\alpha 2\beta 1$ ja CT16 lupaavina melanoomabiomarkkereina. Tutkimus sisältää myös ensimmäisen toiminnallisen tutkimuksen CT16 antigeenistä.

Avainsanat: Kollageenireseptori-integriini, $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, $\alpha 11\beta 1$, syöpä/kives antigeeni, CT16, eturauhassyöpä, melanooma, ilmentyminen

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SAMMANFATTNING

Globalt sett är prostatacancer den näst vanligaste cancerformen hos män. Melanom, som är den mest elakartade hudcanceren, är den cancerform som ökar snabbare än någon annan cancerform. I Finland diagnostiseras varje år mer än 5000 prostatacancerfall och 1200 melanomfall. Genom att erhålla bredare kunskap om genexpressionsförändringar och deras möjliga konsekvenser under cancerutvecklingen kan man utöka förståelsen för cellernas funktioner. Min avhandling fokuserar på integriner och cancer testikel antigener (CT antigener, *eng.* cancer testis antigens), samt deras expressionsnivåer och inverkan på prostatacancer och melanom. Integriner är heterodimeriska transmembranreceptorer som reglerar viktiga cellulära processer som celltillväxt, celldifferentiering och cellmigration. CT antigener är cancerspecifika och immunogena proteiner som expresseras i många olika cancertyper. Deras distribution i normala vävnader är främst begränsad till testiklar. På grund av dessa egenskaper är både integriner och CT antigener ideala för cancerforskning.

Det huvudsakliga målet med min doktorsavhandling var att studera expressionsnivåerna av de kollagenbindande integrinerna $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$ och $\alpha 11\beta 1$ och CT16 i prostatacancer och melanom. I avhandlingen utvärderades också den prognostiska och diagnostiska signifikansen av de kollagenbindande integrinerna och CT antigenen CT16, och den biologiska rollen av CT16 studerades. För övrigt finns det få expressionsstudier av de kollagenbindande integrinerna och CT16 i prostatacancer och melanom. CT16:s biologiska roll är helt okänd.

I denna avhandling noterades en signifikant $\alpha 2$ - och $\alpha 11$ -expressionsförändring i prostatacancer. Expressionsnivån av $\alpha 2$ minskade under prostatacancerens utveckling, och expressionsnivån av $\alpha 11$ var förhöjd i dåligt differentierade prostatacancer vävnader jämfört med friska vävnader. Förhöjda $\alpha 2$ -expressionsnivåer i de metastaserande melanomvävnaderna hos melanompatienter var förenade med en signifikant sämre livslängdsprognos efter cancerdiagnosen. I denna avhandling kunde man även presentera CT16 som en melanomantigen med anti-apoptotiska funktioner. CT16 och $\alpha 2$ kan också föreslås som specifika och lovande biomarkörer för metastaserande melanom. Avhandlingen presenterar också den första funktionella studien av CT16.

Nyckelord: Kollagenbindande integriner, $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, $\alpha 11\beta 1$, cancer testikel antigener, CT16, prostatacancer, melanom, expression

TABLE OF CONTENTS

ABBREVIATIONS	1
LIST OF ORIGINAL PUBLICATIONS.....	2
1. INTRODUCTION.....	3
2. REVIEW OF LITERATURE	4
2.1 PROSTATE CANCER	4
2.1.1 Biomarkers for prostate cancer	4
2.1.2 New drugs for prostate cancer	6
2.2 MELANOMA	7
2.2.1 Biomarkers for melanoma	7
2.2.2 New drugs for metastatic melanoma.....	9
2.3 INTEGRINS.....	10
2.3.1 Integrins in cancer.....	11
2.3.2 The collagen binding integrins	13
2.3.2.1 <i>Integrin $\alpha 161$</i>	13
2.3.2.2 <i>Integrin $\alpha 261$</i>	14
2.3.2.3 <i>Integrin $\alpha 1061$</i>	15
2.3.2.4 <i>Integrin $\alpha 1161$</i>	16
2.3.3 Integrins in prostate cancer	16
2.3.4 Integrins in melanoma	17
2.3.5 Integrins in cancer therapy	18
2.4 CANCER TESTIS ANTIGENS	19
2.4.1 The families of Cancer Testis antigens	20
2.4.1.1 <i>CT-X antigens and non-X CT antigens</i>	20
2.4.2 Cancer Testis antigen 16	20
2.4.2.1 <i>Other members of PAGE- family</i>	21
2.4.3 Regulation of Cancer Testis antigen-expression	22
2.4.4 Cancer Testis antigens in normal tissues	23
2.4.5 Cancer Testis antigens in cancer	23
2.4.5.1 <i>Cancer Testis antigens in cancer therapy</i>	24
2.4.5.2 <i>Expression of Cancer Testis antigens during cancer progression</i>	24
2.4.5.3 <i>Function of Cancer Testis antigens in cancer</i>	25
2.4.6 Cancer Testis antigens in prostate cancer.....	26
2.4.7 Cancer testis antigens in melanoma	26
3. AIMS OF THE STUDY.....	28
4. MATERIALS AND METHODS	29
4.1 Cancer cell lines (I,III, IV) and stable cell lines (IV)	29
4.2 Tissue and serum samples	29
4.2.1 Prostate cancer tissue samples (I, III).....	29
4.2.2 Advanced melanoma samples (II-IV).....	29
4.2.3 Serum samples from advanced melanoma patients (III)	30
4.3 Antibody for CT16 (III, IV)	31
4.4 Total RNA isolation and cDNA synthesis (I-IV).....	31
4.5 Real Time quantitative Reverse Transcription PCR (I-IV)	31
4.6 RNA interference (IV)	32
4.7 Western blotting (III-IV).....	32
4.8 Immunohistochemistry and Immunofluorescence (I, II and IV)	33
4.9 Time-resolved Fluoroimmunoassay (III)	34
4.10 Transcriptome analysis (IV).....	34
4.11 Cell proliferation assays and real time monitoring of cells (IV)	35
4.12 p53 conformation and stability analyses (IV)	35
4.13 Histone-H3-acteylation and DNA-methylation specific immunoprecipitaiton analyses (IV)	36

4.14 Preparation and analysis of nuclear and cytosolic fractions (IV)	36
4.15 Statistical and Data analysis (I-IV)	36
5. RESULTS	38
5.1 Expression of collagen binding integrins in cancer cell lines, prostate cancer and advanced melanoma (I,II)	38
5.1.1 mRNA expression of collagen binding integrins in cancer cell lines	38
5.1.2 Levels of integrin $\alpha 2\beta 1$ decrease in parallel with progression of prostate cancer	39
5.1.3 The expression of integrin $\alpha 11\beta 1$ is increased in prostate cancer	40
5.1.4 $\alpha 1$ and $\alpha 10$ mRNA expression are increased in individual prostate cancer cases	40
5.1.5 Androgen receptor correlates with integrin $\alpha 2\beta 1$	41
5.1.6 mRNA expression of $\alpha 1$ and $\alpha 2$ were significantly higher than of $\alpha 11$ in advanced melanoma	41
5.1.7 Impact on survival in advanced melanoma patients with high or low $\alpha 1$ -, $\alpha 2$ - and $\alpha 11$ mRNA	42
5.1.7.1 <i>Integrin $\alpha 2\beta 1$ has great impact on overall survival in advanced melanoma</i>	42
5.1.7.2 <i>Low levels of $\alpha 1\beta 1$ predict enhanced survival after beginning of treatment and first metastasis</i>	42
5.1.7.3 <i>Low levels of $\alpha 11\beta 1$ predict enhanced survival after beginning of treatment and first metastasis</i>	43
5.1.7.4 <i>Lower levels of $\alpha 2\beta 1$ and $\alpha 11\beta 1$ correlated with diffuse expression of Bcl-2</i>	43
5.2 Expression of Cancer Testis Antigen 16 (CT16) in cancer cell lines, prostate cancer and advanced melanoma (III, IV)	43
5.2.1 CT16 is not expressed in prostate cancer	44
5.2.2 CT16 is associated with melanoma	44
5.2.2.1 <i>CT16 is frequently expressed in tissues of advanced melanoma</i>	44
5.2.2.2 <i>Cellular location of CT16</i>	45
5.2.3 CT16 is frequently expressed in sera of advanced melanoma	45
5.2.3.1 <i>CT16 in sera during melanoma treatment</i>	46
5.2.4 The biological role of CT16	47
5.2.4.1 <i>CT16 inhibits drug induced apoptosis</i>	47
5.2.4.2 <i>CT16 regulates genes associated with survival</i>	48
5.2.4.3 <i>CT16 is a negative regulator of DKK-1 in melanoma</i>	49
5.2.4.4 <i>CT16 is independent of p53 and DNA methylation</i>	50
6. DISCUSSION	51
6.1 Collagen binding integrins in prostate cancer	51
6.1.1 Collagen binding integrins were frequently expressed in sarcoma cell lines	51
6.1.2 Integrin $\alpha 2\beta 1$ is the main collagen receptor in cancer cell lines	51
6.1.3 The expression levels of $\alpha 2\beta 1$ and $\alpha 11\beta 1$ are altered during the progression of prostate cancer	52
6.1.4 AR - a positive regulator of $\alpha 2\beta 1$	53
6.2 Collagen binding integrins in melanoma	54
6.2.1 Integrin $\alpha 2\beta 1$ as a potential prognostic biomarker for advanced melanoma	54
6.2.2 Integrin $\alpha 11\beta 1$ as an interesting marker for advanced melanoma	55
6.3 CT16 in melanoma	55
6.3.1 CT16 is a common melanoma CT antigen	55
6.3.2 CT16 is detected in the sera of melanoma patients	56
6.3.3 CT16 as a melanoma biomarker	56
6.4 The biological role of CT16	57
6.4.1 CT16 regulates its target genes in a p53 independent manner	58
7. CONCLUSIONS AND FUTURE PROSPECTS	59
8. ACKNOWLEDGEMENTS	61
9. REFERENCES	63
10. ORIGINAL PUBLICATIONS I-IV	79

ABBREVIATIONS

AR	Androgen Receptor
AZA	5-aza-2-deoxycytidine
BAGE	B Melanoma Antigen
BIRC5	Baculoviral IAP repeat containing 5
BPH	Benign Prostatic Hyperplasia
CAF	Cancer Associated Fibroblasts
CRPC	Castration Resistant Prostate Cancer
CT	Cancer/Testis
CT16	Cancer/Testis antigen 16
CTL	Cytotoxic T Lymphocytes
CTL-4	Cytotoxic T-lymphocyte-associated Antigen 4
DKK-1	Dickkopf-1
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EMT	Epithelial-Mesenchymal Transition
ERK	Extracellular signal-Regulated Kinases
ESMO	European Society for Medical Oncology
FABP7	Fatty Acid Binding Protein 7
FDR	False Discovery Rate
GAGE	G antigen
HRP	Horse Radish Peroxidase
IDP	Intrinsically Disordered Proteins
IFN	Interferon
IGF	Insulin-like Growth Factor
IHC	Immunohistochemistry
IL-8	Interleukin-8
LDH	Lactate Dehydrogenase
MAGE	Melanoma Associated Antigen
MAPK	Mitogen-activated protein kinases
MART-1/Melan-A	Melanoma Antigen Recognized by T cells
MHC	Major Histocompatibility Complex
MIA	Melanoma Inhibitory Activity
MIU	Million International Units
MMP	Matrix Metalloproteinases
mRNA	Messenger Ribonucleic Acid
MT2A	Metallothionein 2A
NSCLC	Non-Small Cell Lung Cancer
NY-ESO-1 New York	Esophageal Squamous Cell Carcinoma-1
PAGE	Prostate Associated Gene
PHI	Prostate Health Index
PIN	Prostatic Intraepithelial Neoplasia
PSA	Prostate Specific Antigen
PCSC	Prostate Cancer Stem Cells
PSMA	Prostate-Specific Membrane Antigen
PTEN	Phosphatase and Tensin homolog
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
S100B	Calcium Binding Protein B
SSX	Synovial Sarcoma X
TGF- β	Transforming Growth Factor
VEGF	Vascular Epidermal Growth Factor
WHO	World Health Organization
XAGE	X Antigen

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-IV.

- I Mirtti T, **Nylund C**, Lehtonen J, Hiekkänen H, Nissinen L, Kallajoki M, Alanen K, Gullberg D, Heino J. 2006. Regulation of prostate cell collagen receptors by malignant transformation. *International Journal of Cancer* **118**(4):889-98.
- II Vuoristo M, Vihinen P, Vlaykova T, **Nylund C**, Heino J, Pyrhönen S. 2007. Increased gene expression levels of collagen receptors integrins are associated with decreased survival parameters in patients with advanced melanoma. *Melanoma Research* **17**(4):215-223
- III Rappu P, **Nylund C**, Ristiniemi N, Kulpakko J, Vihinen P, Hernberg M, Mirtti T, Alanen K, Kallajoki M, Vuoristo MS, Pyrhönen S, Heino J. 2011. Detection of melanoma-derived cancer-testis antigen CT16 in patient sera by a novel immunoassay. *International Journal of Cancer* **128**(10):2382-2392.
- IV **Nylund C**, Rappu P, Pakula E, Heino A, Laato L, Elo LL, Vihinen P, Pyrhönen S, Owen GR, Larjava H, Kallajoki M, Heino J. 2012. Melanoma-associated cancer-testis antigen 16 (CT16) regulates the expression of apoptotic and antiapoptotic genes and promotes cell survival. *PLoS One* **7**(9) e45382

1. INTRODUCTION

Prostate cancer, which is predominately an adenocarcinoma, develops in the prostate gland and belongs to the most common cancer types. Melanoma is derived from the pigment producing melanocytes and is the most fatal type of skin cancers. Both diseases cause major health issues worldwide. Prostate cancer is listed as the second leading cause of cancer death while metastatic melanoma is among the most aggressive malignancies. Both diseases are known to have a highly heterogeneous nature. Prostate cancer is characterized by numerous genetic alterations and various progression paths, which can range from very mild slow-growing malignant cells to fast aggressively-growing malignant cells or to castration-resistant prostate cancer cells. Melanoma can metastasize rapidly and is characterized as a very highly mutated type of cancer which easily develops drug resistance. Therefore, it is very critical to develop highly specific diagnostic, predictive biomarkers and effective drugs for prostate cancer and melanoma.

Integrins are one of the most critical cell receptors that bind to the extracellular matrix through collagen, fibronectin and laminin. Integrins provide vital cell signalling such as cell proliferation, differentiation, survival and migration. During abnormal conditions like malignancies, the expression of integrins can get altered, which can for example be upregulations or downregulations of an integrin. The knowledge on alterations in the integrin expression is required, to better understand their importance in the malignant transformation and progression. Cancer testis antigens are considered as novel biomarkers and therapy targets due to their cancer-specific expression and their immunogenicity. However, the expression frequency of cancer testis antigens can be very variable according to cancer type and type of cancer testis antigen.

In this thesis, the expression of collagen binding integrins ($\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$ and $\alpha11\beta1$) and cancer testis antigen CT16 were investigated in human metastatic melanoma and human prostate cancer to characterize their expression pattern, their role and to evaluate their significance as potential biomarkers. Genes regulated by CT16 were also studied in this thesis.

2. REVIEW OF LITERATURE

2.1 PROSTATE CANCER

Prostate cancer is the most common cancer among men in Finland, other Western European countries and North America. In Finland, 5043 new cases of prostate cancer and 854 deaths due to prostate cancer were documented in 2013. Prostate cancer, predominantly in its advanced stage, is listed as the second most common cause of death by cancer in men after lung cancer (www.canceregistry.fi, Finnish Cancer Registry). Prostate cancer metastasizes especially to the bone, lymph nodes, lungs and liver (Bubendorf et al. 2000; Andriole et al. 2012). Even though the majority of prostate cancer cases are classified as slow growing, it is important to distinguish the aggressive prostate carcinomas from the slow growing carcinomas. This classification is vital since it is crucial to provide the proper treatment to the right patient group. The highest risk factor for prostate cancer is age. In Finland, most cases are diagnosed between the ages of 65-74 years. Prostate cancer is graded by the Gleason grading system, based on morphological patterns (Gleason and Mellinger 1974). The Gleason grades range from 1 to 5. Grade 1 represents the most well differentiated prostate carcinomas and grade 5 is associated with the poorest differentiation.

Screening for Prostate Specific Antigen (PSA) from serum is the most common method for diagnosing and monitoring the progression of prostate cancer (Catalona et al. 1991). Even though PSA screening is frequently used, it has disadvantages and controversial issues (Hayes and Barry 2014). The screening can be unreliable, especially in the early phase of prostate cancer. In addition to prostate cancer, elevated PSA values can be due to Benign Prostatic Hyperplasia (BPH), prostatitis and urinary retention (Pezaro, Woo, and Davis 2014). Moreover, routine PSA screening has led to overdiagnosis and overtreatment (Tefekli and Tunc 2013). Additionally, two big trials ERSPC (European Randomized Screening for Prostate Cancer) and PLCO (Prostate, Lung, Colorectal and Ovarian screening trial) gave conflicting results about the usefulness of PSA screening. ERSPC indicated that routine PSA screening lowered prostate cancer mortality significantly: by 20 % compared to the control group, which was not screened. PLCO found no difference in mortality between the PSA screened patients and the patients who had no PSA screening (Andriole et al. 2012; Schroder et al. 2009).

2.1.1 Biomarkers for prostate cancer

In addition to PSA, other diagnostic or prognostic factors biomarkers for prostate cancer are available (examples listed in table 1). They are either serum-, urine- or biopsy-based biomarkers. However, since prostate cancer is often multifocal and known to be histologically and biologically heterogeneous (Andreou and Cheng 2010), the diagnosis and the evaluation of prognosis can be challenging based on the biomarker expression in biopsy sample. Serum based PHI (Prostate health index) and urine based PCA-3 (Prostate cancer antigen 3) are two examples of biomarkers, which are both approved by EU and FDA. PHI combines three forms of PSA (formula: $[-2] \text{ proPSA/free PSA} \times \sqrt{\text{total PSA}}$) and it increases the diagnostic accuracy for patients with 2-10 ng/ml of total PSA (Ferro et al. 2013). PHI improves the specific characterization of a

clinically important tumor and is therefore thought to reduce unnecessary biopsies and enhance the predictive power of disease progression during active surveillance (Loeb and Catalona 2014). PCA-3 has shown to be upregulated in 95 % of the primary prostate cancer tumors. It is beneficial when considering re-biopsies in follow-up patients with a previous negative biopsy (Durand et al. 2011). In addition, urine PCA-3 levels may also predict which patients need a MRI (magnetic resonance imaging) scan, since PCA-3 levels correlate significantly with prostate cancer-outcome from the scanning (Leyten et al. 2013).

Among the most promising biomarkers for prostate cancer are PSMA (Prostate-specific membrane antigen), uPA (urokinase Plasminogen Activator), loss of PTEN (Phosphatase and Tensin homolog) and TMPRSS2:ERG (Transmembrane protease serine 2-ETS related gene: v-ets erythroplastosin virus E26 oncogene homolog) (Dimakakos, Armakolas, and Koutsilieris 2014; Salagierski and Schalken 2012).

Table 1. Biomarkers for Prostate Cancer

Biomarker	Gene	Function of protein	Useful for diagnosis or prognosis	References
Total PSA	KLK3	Androgen regulated serine protease cleaving semenogelins	Diagnosis	Catalona et al. 1991
PHI Prostate Health Index	KLK3	Same as above	Diagnosis and Prognosis Predicts high grade cancer and benefits decision of biopsy	Loeb and Catalona 2014
PCA-3	PCA3	Non- protein coding RNA modulating androgen receptor signaling	Diagnosis Benefits decision of re-biopsy	Durand et al. 2011
TMPRSS2:ERG	TMPRSS2 + ERG	Fusion gene disrupts androgen signaling and mediates cell invasion and migration	Prognosis: high expression = worse prognosis/ (or longer survival after androgen deprivation therapy)	Demichelis et al.2007, Hagglof et al. 2014, Tian et al. 2014, (Graff et al 2015)
uPA	PLAU	Serine protease cleaving plasminogen to plasmin that degrades ECM and promotes metastasis and angiogenesis	Prognosis: higher expression = worse prognosis	Kumano et al. 2009
PSMA	FOLH1	Transmembrane glycoprotein with folate hydrolase and endocytic function	Prognosis: higher expression = worse prognosis	Ross et al. 2003
Ki67	MKI67	Nuclear marker of proliferation	Prognosis: higher expression = worse prognosis	Sulik et al. 2002
PTEN	PTEN	inhibits cell growth and survival by suppressing the P13K/ AKT/ mTOR signaling pathway	Prognosis: loss of PTEN = worse prognosis	Mithal et al 2014
IL-6	IL6	Activates AR receptor causing resistance	Prognosis: higher expression = worse prognosis	Nguyen et al. 2014 Feng et al. 2009

The fusion gene TMPRSS2:ERG is an IHC or urine based biomarker and present in approximately 50 % of prostate cancer cases. This marker is associated with higher Gleason grades, aggressive prostate cancer, shorter survival and androgen independency (Demichelis and Rubin 2007; Yu et al. 2010; Hagglof et al. 2014; Tian et al. 2014). The sensitivity of prostate cancer diagnosis increased when urinary TMPRSS2:ERG and PCA-3 levels were combined with analysis of PSA levels in serum (Salami et al. 2013). However, some studies indicate that TMPRSS2:ERG predicts longer survival after androgen deprivation therapy or has no relation to the outcome (after radical prostatectomy) (Pettersson et al. 2012; Graff et al. 2015). However, the presence of TMPRSS2:ERG and the loss of PTEN are the most frequent genomic alterations in prostate

cancer and are often seen together. These alterations predict a five times higher risk of capsular penetration compared to their absence (Nagle et al. 2013).

PSMA is a prognostic biomarker and correlates with tumor stage, biochemical recurrence and aneuploidy in primary prostate cancer. PSMA overexpression is also observed in androgen independent cancer (Ross et al. 2003; Ristau, O'Keefe, and Bacich 2014). Overexpression of the serine protease uPA, which is a degrader of the extracellular matrix (ECM), is associated with high Gleason grade and lymph node metastases. uPA is suggested as a biochemical recurrence marker for patients who have undergone radical prostatectomy (Kumano et al. 2009). The ratio of uPA/PAI-1 (inhibitor of uPA) is significantly higher in prostate cancer compared to BPH (Akudugu, Serafin, and Bohm 2015; Bohm et al. 2013).

General cancer markers associated with prostate cancer are for example IL-6 (Interleukin-6), proliferation marker Ki67 and loss of PTEN. They are linked with higher Gleason grades, aggressive tumor types or poor prognosis (Sulik and Guzinska-Ustymowicz 2002; Mithal et al. 2014). IL-6 is associated with regulation of epithelial-mesenchymal transition (EMT), invasion to bone and transition from hormone-dependent to castration resistant prostate cancer (CRPC) (Nguyen, Li, and Tewari 2014).

Even though new biomarkers are being discovered, there is still a continuing need to improve the characterization of diagnostic and prognostic markers for prostate cancer. Accurate markers are needed to predict the progression rate, the metastatic onset and to determine the spread of the metastases. With the aid of more specific markers, common issues such as overtreatment, unnecessary biopsies and delays in the treatment of aggressive prostate cancer could be avoided (Klotz 2013).

2.1.2 New drugs for prostate cancer

The standard treatment of advanced prostate cancer (local or metastasized) is to reduce the levels of testosterone. However, most hormone dependent prostate cancers will eventually develop resistance to the androgen deprivation therapy and the disease is then classified as castration resistant prostate cancer (CRPC). During the last five years several new drugs are have become available for CRPC (Tucci, Scagliotti, and Vignani 2015).

The drug abiraterone acetate is an androgen synthesis inhibitor and has increased the median survival time in metastatic CRPC patients with four months compared to placebo-prednisone (immunosuppressant) group (de Bono et al. 2011). The androgen receptor antagonist enzalutamide blocks the receptor mediated signaling and enhances the median overall survival with about five months (Scher et al. 2012). Sipuleucel-T was recently approved in EU and is the first FDA-EU approved therapeutic cancer vaccine. The vaccine is designed to start an immune response to prostatic acid phosphatase (PAP) in the asymptomatic and metastatic CRPC patients. It prolongs the median survival with 4 months (Cheever and Higano 2011).

2.2 MELANOMA

Melanoma is derived from melanocytes and develops predominantly in the skin but can also occur in eye and intestine. Melanoma is one of the most rapidly increasing cancer types in Finland and in many other western countries. In Finland in year 2014, 1362 new melanoma diagnoses were registered, out of which 49 % were in women (Finnish Cancer Registry). The highest risk factor for developing melanoma is intense exposure of UV radiation such as severe sunburns during childhood (Whiteman, Whiteman, and Green 2001). Additional factors are certain skin pigmentation phenotypes, atypical nevi and family histories (Gandini et al. 2005).

Cutaneous malignant melanoma develops in the basal layer of the epidermis and tends to grow horizontally before expanding vertically (Urso 2004). The malignant cells cross the basal membrane and enter the dermis/subcutaneous layer, where they metastasize via lymph/blood vessels. Typical sites of metastasis are the lungs, liver, bone and brain. The stage of melanoma is commonly determined by the pathological TNM (Tumor, Node and Metastases) staging system, ranging from stage 0 to IV. T represents the thickness of the primary tumor and is measured with the Breslow scale. The tumor thickness is still the most important progression factor in melanoma. N describes the number of nearby lymph nodes and M represents the presence of metastases. Additionally, mitotic rate (number of mitoses/mm²), ulceration and lactate dehydrogenase (LDH) levels have been integrated into the TNM staging system as prognostic factors (Balch et al. 2001; Balch et al. 2009). Melanoma stage 0 or melanoma *in situ* is characterized as a premalignant state restricted to the epidermis. Melanoma stage IV is the most advanced type, characterized by metastases found in distant lymph nodes as well as in other organs of the body.

Even though melanoma is usually easier to detect than other cancer types, melanoma is aggressive and likely to metastasize. Early detection is very critical, since metastatic melanoma is unpredictable and difficult to treat and becomes quickly resistant to drugs (Somasundaram, Villanueva, and Herlyn 2012). The histological diagnosis of melanoma can also be a challenge, since it can be difficult to distinguish benign melanocytic nevi from malignant cells (Weinstein et al. 2014). The prognosis of patients with metastatic melanoma is poor and the median survival time is about 8-10 months (Tas 2012).

2.2.1 Biomarkers for melanoma

Since the incidence of melanoma is increasing and because melanoma is characterized as a very severe type of cancer when metastasized, there is an urgent need for more sensitive and more specific melanoma biomarkers. There is especially a need of biomarkers that accurately predict progression patterns and early metastasis, and biomarkers for treatment choices and follow-up of melanoma patients (Vereecken et al. 2012).

Today, LDH is the most clinically used biomarker and the only biomarker included in the TNM staging system. Elevated LDH levels are associated mainly with stage IV melanoma, liver metastasis and shorter survival (Weide et al. 2012). Examples of melanoma biomarkers are listed in table 2. Another well-established and independent prognostic serum/IHC biomarker is

S100B (S100 calcium binding protein B), linked with stages III-IV, tumor recurrence, response to chemo-immunotherapy and poor survival (Smit et al. 2008; Kruijff et al. 2009; Hamberg et al. 2003). S100B has shown to be more sensitive for the early detection of distant metastasis than LDH (Egberts et al. 2009). The ESMO (European Society for Medical Oncology) guidelines state that S100 protein is the most preferable serum marker for follow-up in patients, because of its high specificity in the prediction of melanoma progression (Dummer et al. 2012). However, both LDH and S100B are poor markers for early stages of melanoma and loco-regional metastases. Another disadvantage is elevated LDH levels due to other conditions than melanoma. Elevated LDH levels can be caused by tissue injury, inflammation or myocardial infections. The levels of S100B have been shown to be increased after stroke or head injury and in neurodegenerative diseases (Egberts et al. 2009).

Further IHC based biomarkers for diagnosing melanoma include the pre-melanosome protein HMB-45 (human melanoma black 45), Melan-A (melanoma antigen, MART-1), tyrosinase and CSPG4 (chondroitin sulfate proteoglycan 4). HMB-45 is melanoma specific and present in 50-70 % of the melanoma cases. It has lower sensitivity in metastases than primaries (Orchard 2000). Melan-A has recently shown a high expression frequency in melanomas (93 %), but can be positive in about 2 % of the non-melanocytic cancer cases (Jing, Michael, and Theoharis 2013). Other Melan-A studies have reported an expression frequency around 75 % in melanomas (Miettinen et al. 2001; Reinke et al. 2005). Melan-A and HMB-45 as well as many other melanoma biomarkers are expressed very poorly in desmoplastic melanomas (Weinstein et al. 2014). CSPG4 on the other hand seems to be sensitive for desmoplastic melanoma. The expression frequency of CSPG4 was 95% in desmoplastic primary lesions (Goto et al. 2010).

Apart from the most recommended prognosis biomarkers LDH and S100B, there are other prognostic marker candidates like Ki-67, metallothioneins and MIA (Melanoma Inhibitory Activity). They are all associated with a worse prognosis when elevated. Enhanced levels of proliferation marker Ki-67 are linked with increased tumor thickness and ulceration (Ladstein et al. 2010). Metallothioneins are suggested as melanoma progression indicators for primary cutaneous melanoma in thin lesions (< 1.5 mm) (Weinlich et al. 2003). Metallothioneins correlate significantly with tumor-associated macrophages, which are known to promote melanoma progression, metastases and poor prognosis (Emri et al. 2013). The serum/IHC marker MIA is a secreted protein produced by malignant melanocytes. MIA was first proposed to suppress melanoma, hence its name, Melanoma Inhibitory Activity (Blesch et al. 1994). MIA has shown to support aggressive melanoma phenotype by promoting cell migration and local invasion through interaction with integrins $\alpha 4\beta 1$ and $\alpha 5\beta 1$ (Schmidt and Bosserhoff 2009). MIA interacts directly with $\alpha 4\beta 1$ and $\alpha 5\beta 1$ and reduces their activity and MAPK signaling (Bauer et al. 2006). Studies have also reported that MIA blocks $\alpha 4\beta 1$ on the leukocytes to promote immunosuppression by decreasing the cytotoxic activity of lymphokine-activated killer cells and the leukocyte proliferation (Sandru et al. 2014). Inactivation of MIA with peptides has decreased the numbers of melanoma metastases *in vivo* (Schmidt, Riechers, and Bosserhoff 2013). Enhanced MIA levels in serum have recently been suggested to be an independent prognostic factor for tumor relapse in melanoma stages I and II. 66 % of primary melanoma patients with high MIA levels (>9.4 ng/ml) had tumor relapse, while only 5 % of the patients with low MIA

levels had tumor relapse (Sandru et al. 2014). Both MIA and S100B may function as markers for BRAF inhibitor treatment in metastatic melanoma (Sanmamed et al. 2014).

Table 2. Biomarkers for melanoma

Biomarker	Gene	Function	Useful for diagnosis or prognosis	References
LDH	ldh	Involved in cellular respiration	Diagnosis and Prognosis	Weide et al. 2012
S100B	S100B	Associated to cell cycle, growth and differentiation	Diagnosis and Prognosis	Weide et al. 2012
HMB-45	PMEL	Forms matrix of melanosomes	Diagnosis	Orchard et al. 2000
Melan-A/ MART-1	MLANA	Involved in melanosome biosynthesis	Diagnosis	Reinke et al. 2005
Tyrosinase	TYR	Involved in melanin synthesis	Diagnosis	Reinke et al. 2005
CSPG4	CSPG4	Enhances cell motility and growth	Diagnosis	Goto et al. 2012
MIA	MIA	Autocrine growth factor	Prognosis: higher expression = worse prognosis	Schmidt et al. 2013
Ki-67	MKI-67	Nuclei marker for cell proliferation	Prognosis: higher expression = worse prognosis	Ladstein et al. 2010
Metallothionein	MT	Homeostatic mechanism and antioxidants	Prognosis: higher expression = worse prognosis	Weinlich et al. 2009 and Emri et al. 2013

Melanoma is a highly mutated cancer type with great heterogeneity between tumors. This makes melanoma a complex and challenging disease to study (Somasundaram, Villanueva, and Herlyn 2012). The BRAF^{V600E} (B-raf kinase mutation) is the most common melanoma mutation and it occurs in 40-60 % of all melanoma cases. The active oncogenic mutation keeps the mitogen-activated protein kinases (MAPK) pathway in a permanent active state promoting cell proliferation and survival (Davies et al. 2002). Other genetic alterations in melanoma are found in NRAS, AKT3 and PTEN (Bertolotto 2013). Due to the identification of the BRAF mutation, the first specific treatment for advanced melanoma has become available, and has improved the survival (Chapman et al. 2011). However, the survival improvement is limited since resistance occurs at a median time of six months (Chapman 2013).

2.2.2 New drugs for metastatic melanoma

The first therapies for specifically treating metastatic melanoma were approved by the EU in 2012. They are based on targeting the mutated BRAF or the cytotoxic T-lymphocyte-associated Antigen 4 (CTLA-4). The BRAF inhibitor (vemurafenib) interrupts the BRAF/MEK/ERK (MAPK) pathway, leading to apoptosis and a decrease in cell proliferation. In the phase III trial, 84 % of the advanced melanoma patients were alive in the vemurafenib group and 64 % in the dacarbazine group six months after initiating the therapy (Chapman et al. 2011). The monoclonal antibody treatment (ipilimumab) upregulates and activates cytotoxic T-lymphocytes to destroy tumor cells by blocking the receptor CTLA-4 (Hodi et al. 2010). Recently, inhibitors of the kinases MEK-1 and MEK-2 (trametinib) were approved as drugs for metastatic melanoma in the EU. MEK-1 and MEK-2 are downstream targets of BRAF in the MAPK pathway (Ribas et al. 2014).

Despite the recent emergence of promising treatments for advanced melanoma, a rapidly developing drug resistance is still a major issue in combatting the disease, and the mortality of metastatic melanoma remains high. Patients treated with BRAF inhibitors develop resistance after a median progression-free survival of approximately seven months (Hartsough and Aplin 2013). A combination of BRAF and MEK inhibitors has extended survival by delaying the drug resistance (Ribas et al. 2014). The resistance is linked to the reactivation of the MAPK pathway by enhanced phosphorylation of ERK and mutations in MEK-1 and NRAS (Trunzer et al. 2013). Resistance may also be explained by the activation of the PI3K (phosphatidylinositol-3 kinase) /PTEN–AKT–mTOR pathway (Shi et al. 2014). The (re)activation of the pathways and other resistance mechanisms seem to be all associated with the elevated and persistent formation of eIF4F (Eukaryotic Translation Initiation Factor 1A) complexes. The eIF4F complexes are suggested to act as a resistance biomarkers and possible therapeutic targets in both anti-BRAF^{V600E} and in anti-MEK resistance (Boussemart et al. 2014).

By further investigating the molecular mechanisms and alterations in protein expression during cancer progression, additional important molecular/drug targets or biomarkers are likely to be discovered. Two protein families, which have been listed as potential and interesting biomarkers/drug targets for melanoma, are integrins and cancer testis antigens (Vereecken et al. 2012).

2.3 INTEGRINS

Integrins are a large family of transmembrane receptors, which are connected to the ECM by ligand binding and are linked to the actin cytoskeleton through intracellular tails. Integrins also mediate cell-cell interactions (Hynes 2002). Integrins are heterodimers composed of one α -chain and one β -chain with non-covalent binding links. The family consists of 18 α -chains and 8 β -chains, which form 24 distinct integrins. Integrins bind specifically to ECM proteins such as collagens, fibronectins, laminins, vitronectins and osteopontins, and to intracellular and vascular cell adhesion proteins (ICAM-1 and VCAM-1). Integrins can be categorized into subfamilies according to their main ligands, attachment site or their expression in cells (Figure 1). The subfamilies are 1) collagen binding integrins, 2) laminin binding integrins, 3) leukocyte specific integrins (integrins expressed on leukocytes) and 4) RGD (Arg-Gly-Asp) binding integrins, which attach to the tripeptide site in various ECM proteins. Integrins $\alpha 4\beta 1$ and $\alpha 9\beta 1$ form their own small subfamily, based on high sequence homology and shared functions (Palmer et al. 1993). Integrins $\alpha 4\beta 1$ and $\alpha 9\beta 1$ mediate cell migration by binding to VCAM-1, fibronectin and osteopontin (Yokosaki et al. 1999; Ito et al. 2009; Liao et al. 2002). The collagen binding integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$ and $\alpha 11\beta 1$ as well as αD , αE , αL , αM , and αX , are composed of an extra inserted domain (I-domain), located near the aminoterminal of the extracellular α -subunit.

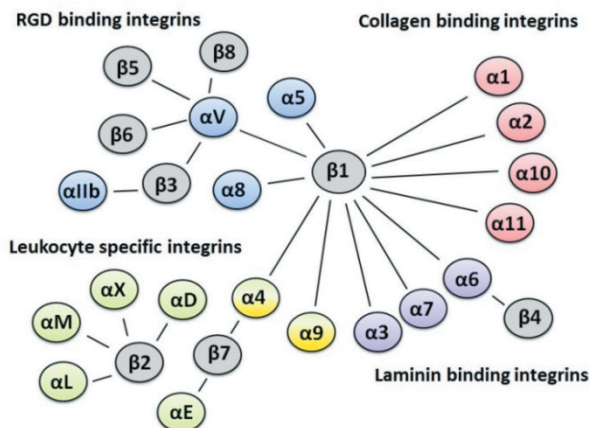


Figure 1. The integrin family

The integrin family consists of 24 heterodimers, which can be divided into several subfamilies. The integrin family is composed of 18 α -subunits and 8 β -subunits. An integrin is composed of one α -subunit and one β -subunit (Hynes 2002).

The highly conserved I-domain is responsible for binding to specific peptide motifs on the ECM ligand, such as the triple helical GFOGER sequence in collagens (Kamata, Puzon, and Takada 1994; Emsley et al. 2000). During binding, integrins recruit various intracellular signaling proteins and cytoskeletal adapter proteins to their short cytoplasmic tails. This results in the formation of macromolecular assemblies, known as focal adhesions. Through extracellular- and intracellular molecular interactions, command “outside-in”- and “inside-out-signaling. Integrin inhibitors, which prevent the binding of integrins to their main ligand, can command a negative signaling (Madamanchi, Santoro, and Zutter 2014). Integrins are known to activate FAK and Src which mediate cascades such as p38-MAPK pathway and PI3K/AKT pathway. These signaling events control vital cellular functions which make integrins key players in the regulation of the proliferation, adhesion, migration, survival and invasion of cells. Integrins are therefore crucial receptors for processes like initiation-, progression- and invasion of cancer, and for development of metastases (Hood and Cheresch 2002). Integrins are also central players in cardiovascular diseases, neurodegenerative disorders, inflammation, wound healing, and embryonic development (Clemetson and Clemetson 1998; Wu and Reddy 2012).

2.3.1 Integrins in cancer

During cancer progression, tumor cells tend to develop an altered affinity towards ECM molecules. This change can be promoted by alterations in the expression of integrins. A change in the expression profile of an integrin may mean an up- or down-regulation of the integrin, but also a complete absence of a previously expressed integrin or a replacement of one integrin type with another. The integrin expression can vary greatly between cancer types and in

different cancer tissues, but also in different cells. The altered integrin expression may not follow any histological pattern. Some typical changes in integrin expression have been characterized. Integrin $\alpha 2\beta 1$ is generally decreased in breast carcinoma, since the integrin normally contributes strongly to the maintenance of differentiated breast epithelial cells (Zutter, Krigman, and Santoro 1993). The re-expression of $\alpha 2$ in breast cancer cells results in a less malignant phenotype, with epithelioid shaped cells and with decreased adhesion, mobility and invasion properties (Zutter et al. 1995).

Ligand binding-affinity and -specificity may change upon altered integrin expression, which can lead to the dysregulation of cell signaling. This can have a strong impact on the interactions of the cells with ECM (Hood and Cheresch 2002). Cells can become more capable of surviving, proliferating, migrating and invading tissues, while ECM can be remodeled by contraction, degradation or synthesis. Altered integrin expressions can for example promote dysregulation of matrix-metalloproteinases (MMP), serine- and cysteine proteases. They, in turn, start to degrade and change the ECM to get it more accessible for the invading tumor cells. MMP-2 is able to activate the $\alpha V\beta 3$ -mediated adhesion and migration of human melanoma cells by cleaving fibronectin into small fragments (Jiao et al. 2012). Integrins have also been found to contribute to cancer through their critical role in cancer stem cells. The integrin subunits $\alpha 2$ and $\alpha 6$ play important roles in the maintenance and proliferation of cancer stem cells in prostate adenocarcinoma and glioblastoma (Collins et al. 2005; Lathia et al. 2010).

Furthermore, integrins actively engage in cross talk with growth factors including epidermal growth factor (EGF), transforming growth factor beta (TGF β), vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF), and various cytokines and oncogenes during cancer progression (Desgrosellier and Cheresch 2010). Co-operation with growth factors contributes to enhanced activation of the downstream kinase targets, such as Src, MAPK and AKT and may also lead to drug resistance. For instance, in co-operation with IGF, $\beta 1$ -subunits have shown to decrease docetaxel- and ceramide-induced apoptosis in DU-145 prostate cancer cells (Thomas et al. 2010).

The RGD-binding αV -integrins which have a wide array of ligands, (fibronectin/vimentin/osteopontin/laminin) belong to the most investigated integrins in malignancies. αV -integrins are highly upregulated on endothelial cells, where they participate actively in survival and angiogenesis (Weis and Cheresch 2011). αV -integrins mediate also the epithelial-mesenchymal transition (EMT) during cancer progression through TGF- β signaling (Mamuya and Duncan 2012). Antiangiogenic factors like endostatin, tumastatin and arresten, which are all collagen fragments, are known to associate with integrins to decrease the angiogenic process (Weis and Cheresch 2011). Integrin $\alpha V\beta 3$ is associated with bone metastases in breast and prostate cancer (Sloan et al. 2006; McCabe et al. 2007), and with lymph node metastases in melanoma and pancreatic cancer (Nip et al. 1992; Hosotani et al. 2002).

Other well documented integrins in cancer are $\alpha 6\beta 4$, $\alpha 5\beta 1$ and $\alpha 4\beta 1$. Of these integrins, $\alpha 4\beta 1$ and $\alpha 5\beta 1$ are known to actively promote melanoma metastasis (Schlesinger et al. 2014; Arpaia et al. 2012), while $\alpha 6\beta 4$, is known to be lost and replaced by $\alpha 6\beta 1$ in prostate adenocarcinoma (Knox et al. 1994; Nagle et al. 1994). Integrin $\alpha 6\beta 4$ keeps the hemidesmosomal-like structures

intact in normal prostate. Collagen binding integrins also participate in malignancies, but have been studied far less in different cancer types than the previously mentioned integrins.

2.3.2 The collagen binding integrins

Collagen is a fibrous and the most abundant protein in vertebrates. It is a major component in the ECM and provides a tensile and structural strength for the matrix (Mouw, Ou, and Weaver 2014). Currently, 28 different types of collagen have been identified and they can be divided into fibrillar and non-fibrillar collagens (Shoulders and Raines 2009). Collagen type I (fibrillar collagens) is the most abundant collagen and are specifically found in bone, skin, tendon and ligaments (Shoulders and Raines 2009). Collagen type IV, which is common non-fibrillar collagen, is the major stability component of the basement membrane (Poschl et al. 2004).

Four out of 24 integrins are collagen receptors; $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$ and $\alpha 11\beta 1$. Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ exhibit an abundant expression pattern and are present on e.g T-cells, endothelial cells and fibroblasts (Hemler et al. 1985). In contrast, the expression profiles of $\alpha 10\beta 1$ and $\alpha 11\beta 1$ are far more restricted. Integrins $\alpha 10\beta 1$ and $\alpha 11\beta 1$ are mostly present in chondrocytes and fibroblasts, respectively (Camper et al. 2001; Popova et al. 2007). Even though $\alpha 10$ and $\alpha 11$ share greatest homolog identity (42 %) among the collagen binding integrins, they differ in their ligand specificities (Velling et al. 1999). Integrin $\alpha 1\beta 1$ and $\alpha 10\beta 1$ have similar binding properties and exhibit enhanced selectivity towards network forming collagens (collagen type IV, VI), instead of fibril forming collagens (type I-III, V and XI) (Tulla et al. 2001). Integrins $\alpha 2\beta 1$ and $\alpha 11\beta 1$ prefer to adhere to fibril forming collagens. In addition to its preferred ligands, integrin $\alpha 1\beta 1$ recognizes collagen type I, II, III and XIII and $\alpha 2\beta 1$ binds collagen types IV, VI and VII (Kern et al. 1993; Ruggiero et al. 1996; Nykvist et al. 2000; Saelman et al. 1994). At present, knowledge on the latest detected integrins, $\alpha 10\beta 1$ and $\alpha 11\beta 1$, is modest, especially little is known about $\alpha 10$ when compared to $\alpha 1$ and $\alpha 2$. In addition to collagen, $\alpha 1\beta 1$ and $\alpha 2\beta 1$ can bind to laminins, and $\alpha 2\beta 1$ binds to tenascin (Sriramarao, Mendler, and Bourdon 1993; Languino et al. 1989). All four α -subunits of the collagen binding integrins form a heterodimer with the $\beta 1$ -subunit. The $\beta 1$ -subunit is the largest subgroup of integrins and it associates with 12 different α -chains creating adhesions to collagen, fibronectin, laminin, tenascin C and vitronectin (Brakebusch and Fassler 2005).

2.3.2.1 Integrin $\alpha 1\beta 1$

Integrin $\alpha 1\beta 1$ was originally described as Very Late Antigen-1 (VLA-1) due to its delayed expression after T-cell activation (Hemler et al. 1985; Zutter and Santoro 1990). Integrin $\alpha 1\beta 1$ is present mostly in mesenchymal cells, immune cells, fibroblasts, smooth muscles cells and the microvascular endothelial cells of skeletal, skin and brain tissues. It is generally not present in normal epithelia or larger blood vessels (Belkin, Belkin, and Koteliansky 1990; Defilippi et al. 1991). Integrin $\alpha 1\beta 1$ is thought to have an active role in promoting cell proliferation and migration, but is also a negative regulator of collagen synthesis (Pozzi et al. 1998; Ravanti et al. 1999). Cell proliferation and inhibition of collagen synthesis are primarily regulated through Shc-

mediated ERK activation. Migration is dependent on focal adhesions and the activation of p38 MAPK- and the PI3K/AKT pathways (Pozzi et al. 1998; Abair et al. 2008).

Studies report that integrin $\alpha1\beta1$ is dysregulated in cancer. In hepatocellular carcinoma, which is associated with fibrosis in the liver, integrins $\alpha1\beta1$ and $\alpha2\beta1$ are upregulated and they promote the tumor cell migration across the fibrotic matrix in co-operation with TGF- $\beta1$ and EGF (Yang et al. 2003). In colorectal cancer, $\alpha1\beta1$ is significantly upregulated in both tumor and peritumoral stromal cells and is suggested to have a critical role in the cancer progression. About 65 % of the tumor tissues and 90 % of the peritumoral stromal tissues expressed high or moderate levels of $\alpha1\beta1$, while 23 % of the normal epithelia expressed $\alpha1\beta1$ (Boudjadi, Carrier, and Beaulieu 2013). Upregulations have also been identified in gastric carcinoma (Fukuda et al. 2012) and squamous cell carcinoma (Senger et al. 2002).

Integrin $\alpha1\beta1$ cooperates with a mutated oncogenic KRAS to induce the progression of non-small cell lung cancer (NSCLC). An *in vivo* study demonstrated that KRAS^{LA+}/ $\alpha1$ KO chimeric mouse models gained essentially less primary lung tumors as well as less and smaller vascularized tumors with decreased cell proliferation and increased apoptosis than KRAS^{LA+}/ $\alpha1$ WT mice (Macias-Perez et al. 2008).

In addition to $\alpha V\beta3$ and $\alpha V\beta5$, which are linked with angiogenesis, both $\alpha1\beta1$ and $\alpha2\beta1$ have been reported to be active in VEGF-mediated endothelial cell migration and angiogenesis. Antibody inhibition of $\alpha1\beta1$ and $\alpha2\beta1$ in squamous cell carcinoma *in vivo* reduced tumor growth, endothelial migration and angiogenesis in the collagen rich matrix of the skin (Senger et al. 2002). Recently, the $\alpha1$ and $\alpha2$ dual antagonists: vimocin and vidapin (cyclic peptides containing the KTS lysine-threonine-serine-binding motif) were shown to greatly inhibit the proliferation of endothelial cells and VEGF-induced angiogenesis in a vascular membrane assay (Momic et al. 2014). The collagen IV fragment arresten has also suppressed the angiogenic process and the tumor growth by direct interaction with integrin $\alpha1\beta1$ on microvascular endothelial cells and tumor cells *in vivo*. Arresten increased apoptosis by upregulating Bcl-2 and Bcl-xL (Nyberg et al. 2008).

2.3.2.2 Integrin $\alpha2\beta1$

Integrin $\alpha2\beta1$ (Very Late Antigen-2, VLA-2) is expressed mostly in epithelial cells, platelets and fibroblasts. It is also found in endothelial cells and lymphocytes (Zutter, Krigman, and Santoro 1993; Hemler et al. 1985). Integrin $\alpha2\beta1$ participates in hemostasis, thrombosis, inflammation, wound healing and cancer. Integrin $\alpha1\beta1$ and $\alpha2\beta1$ often induce opposite cellular signaling. Unlike $\alpha1\beta1$, integrin $\alpha2\beta1$ is often a stimulator of collagen synthesis and an inhibitor of cell growth (Riikonen et al. 1995; Heino 2000).

Integrin $\alpha2\beta1$ is known to be greatly down-regulated in breast cancer. Integrin $\alpha2\beta1$ is highly expressed in the normal breast epithelium, where it maintains the differentiated cell phenotype. In breast carcinoma, the reduction of $\alpha2\beta1$ decreases the capability of the tissue to form gland-like structures. The loss of $\alpha2$ is also seen in distant metastases in breast cancer (Zutter, Krigman, and Santoro 1993; Ramirez et al. 2011). A transgenic mouse study has shown that epithelial-mesenchymal transitions (EMT) and metastases were found abundantly in $\alpha2$ -KO mice compared to the control mice. However, the primary $\alpha2$ KO-tumors did not differ markedly

in collagen depositions, apoptosis and in tumor proliferation in comparison to the WT primary tumors (Ramirez et al. 2011). Increased levels of the autocrine protein semaphorin-3A has been reported to enhance and stimulate $\alpha 2$ /collagen I binding *in vitro* and suppress cell migration in breast cancer (Pan et al. 2009).

However, in contrast to breast cancer, integrin $\alpha 2\beta 1$ promotes the metastatic process of colorectal cancer. In colorectal cancer, integrin $\alpha 2\beta 1$ - positive cells are thought to migrate specifically to the liver (Yoshimura et al. 2009). This site specificity can partially be due to $\alpha 2\beta 1$ -collagen IV adhesion and focal adhesion kinase (FAK) activation in the sinusoidal blood vessels of the liver. The basement membrane of the vessels is known to express high levels of collagen IV. Integrin $\alpha 2$ /collagen IV-signaling might also be essential for liver metastasis in many other tumor types (Burnier et al. 2011; Yoshimura et al. 2009). The liver specificity is also associated with interactions between $\alpha 2\beta 1$ and cadherin-17. Cadherin-17 is particularly expressed in liver metastases and induces through $\alpha 2\beta 1$ -signaling proliferation of liver metastases. The cadherin modulated integrin $\alpha 2\beta 1$ -signaling enhances the activation of FAK, Ras, ERK and c-Jun kinase which increases cyclin D1 and the colorectal cancer cell proliferation (Bartolome et al. 2014).

2.3.2.3 Integrin $\alpha 10\beta 1$

Integrin $\alpha 10\beta 1$ was identified in 1998 and is characterized as the main collagen binding integrin in chondrocytes in both adult and embryonic cartilage (Camper, Hellman, and Lundgren-Akerlund 1998). Integrin $\alpha 10\beta 1$ is involved in the development of cartilage and is identified as a marker for chondrocyte differentiation. Integrin $\alpha 10$ is observed already at day 11.5 in mouse embryos (Camper et al. 2001; Bengtsson et al. 2001). The regulation of $\alpha 10$ seems to involve the transcription factors AP-2 epsilon and Ets-1 (Wenke et al. 2006). Integrin $\alpha 10\beta 1$ is expressed in the articular cartilage of the vertebral column, joints, trachea and bronchus, as well as in the heart valves and fibrous connective tissues that support skeletal muscles and tendons (Camper et al. 2001). The upregulation of $\alpha 10\beta 1$ during chondrocyte differentiation in mesenchymal stem cells is stimulated by Fibroblast Growth Factor-2 (FGF-2) (Varas et al. 2007). One study has reported that $\alpha 10\beta 1$ is also found in mouse osteoblasts. Retinoblastoma protein (pRb), a known tumor suppressor, is involved in the transcriptional activation of $\alpha 10$ in mouse osteoblasts (Engel et al. 2013).

To date, there are limited studies on $\alpha 10\beta 1$ in cancer. However, data from human genome microarray studies revealed a significant decrease in $\alpha 10$ mRNA levels in melanoma, lung-, bladder-, renal-, colorectal- and breast cancer tissues compared to healthy tissues (Engel et al. 2013). Breast cancer had the largest decrease (fold change) of $\alpha 10$. The cancer cell types with a significant reduction of $\alpha 10$ had also a frequent inactivation of the pRb pathway, reduced levels of $\alpha 7$ and $\alpha 8$ as well as overexpression of $\beta 4$ (Engel et al. 2013). High levels of integrin subunit $\beta 4$ are known to be related to anchorage-independent cell growth, cancer cell migration, metastasis and poor prognosis (Mercurio and Rabinovitz 2001). The altered expression of $\alpha 10$, $\alpha 7$, $\alpha 8$ and $\beta 4$, and the loss of pRb can diminish the important cell-ECM adhesions and induce cancer cell detachment and the metastatic capacity (Engel et al. 2013).

2.3.2.4 Integrin $\alpha 11\beta 1$

Integrin $\alpha 11\beta 1$, which was identified by Lehnert et al. in 1999, is primarily expressed in fibroblasts, embryonic fibroblasts and mesenchymal non-muscle cells. It is found in cartilage, bone, uterus and heart (Lehnert et al. 1999; Velling et al. 1999; Popova et al. 2007). Integrin $\alpha 11\beta 1$ is enriched in the fibroblasts of the periodontal ligament in mice, where it upregulates MMP-13 and cathepsin K to reorganize the matrix and provide a strong force for tooth eruption (Barczyk et al. 2013; Popova et al. 2007).

Integrin $\alpha 11\beta 1$ -expression in fibroblasts is regulated by TGF- $\beta 1$ which is dependent on the transcription factors SMAD and Sp-1 (Lu et al. 2010). Through TGF- $\beta 1$ signaling, $\alpha 11\beta 1$ regulates collagen I-mediated myofibroblast differentiation and promotes collagen synthesis and matrix reorganization (Carracedo et al. 2010; Lu et al. 2014). The differentiation and overabundance of myofibroblasts (cancer-associated fibroblasts, CAF) in stroma are critical factors for the presence of tumor-stromal interactions (Carracedo et al. 2010). CAFs and tumor-stromal interactions are known to promote tumor growth, invasion, metastases, angiogenesis, chemo resistance and poor clinical prognosis (Zhang and Huang 2011). CAFs are connected mostly to tumor growth and invasion in melanoma and prostate cancer as well as with EMT in prostate cancer by secreting cytokines, growth factors and proteases such as ILs, SDF (stromal-derived factor), VEGFs and MMPs (Zhou et al. 2015; Cirri and Chiarugi 2011).

Integrin $\alpha 11\beta 1$ has been suggested as a marker for non-small cell lung cancer (Lu et al. 2014). It is poorly expressed in normal lung specimens. In lung cancer, integrin $\alpha 11$ is specifically localized and enhanced in CAFs in stroma, where it takes part in tumor-stromal interactions. *In vivo* lung cancer studies have shown that $\alpha 11$ promotes tumor growth rate by increasing levels of insulin growth factor-2 (IGF-2) (Zhu et al. 2007). IGF-2 is known to promote the growth of epithelial tumor cells (Hodak et al. 1996). Other tumor-stromal interactions in lung cancer have been observed when $\alpha 11$ enhances the interstitial fluid pressure by contracting the collagen matrix. This results into a stiff stromal matrix which enhances tumorigenicity and metastasis in lung cancer. A stiff stromal matrix is also a difficult environment for chemotherapy. Integrin $\alpha 11\beta 1$ is a possible stromal target for lung cancer therapy and could increase the efficacy of chemotherapy (Lu et al. 2014).

2.3.3 Integrins in prostate cancer

A gene expression array study based on datasets from the Oncomine database, presented integrin pathways as one of the most central factors associated with prostate cancer (Gorlov et al. 2009). However, the functions of integrins and their expression patterns in prostate cancer are not fully understood. Especially, the knowledge about $\alpha 10$ and $\alpha 11$ in prostate cancer is limited.

One study has shown that the normal prostate gland expresses the following α -subunits of integrins: $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$ and αV . The integrins were localized mostly in the basal cells surrounded by laminin, collagen type IV and entactin of the basement membrane (Nagle et al. 1994). Some studies have shown a decrease in $\alpha 2$ as well as $\alpha 3$ -, $\alpha 4$ -, $\alpha 5$ - and $\alpha 6$ - expression in human primary prostate cancer lesions (Bonkhoff, Stein, and Remberger 1993; Nagle et al. 1994). A prostate cancer study has shown that $\alpha 2\beta 1$ is a specific mediator of bone metastases

through collagen type I binding. Collagen type I is the most common bone protein and $\alpha 2\beta 1$ was abundantly expressed in bone metastases rather than in soft tissue metastases or primary prostate cancer lesions (Sottnik et al. 2013). The $\alpha 2$ -collagen I binding activates the RhoC guanosine triphosphatase *in vitro*, which is suggested to mediate the invasion of prostate cancer to bones (Hall et al. 2008). RhoC guanosine triphosphatase participates in the contractions of actin/myosin filaments and can be active in cellular motility.

Interestingly, integrin $\alpha 2\beta 1$ has also been characterized as a marker for prostate epithelial stem cells (PESC) and prostate cancer stem cells (PCSC)/ tumor initiating cells (Collins et al. 2001; Collins et al. 2005). The stem cells were identified and isolated as a phenotype carrying three cell surface receptors; $\alpha 2\beta 1$ /CD44/CD133. The epithelial stem cells and the prostate cancer stem cells in primary tissues had a remarkable potential of self-renewal, proliferation and differentiation. The tumor stem cell population was app. 0.1 % in most of the primary samples and was independent of the Gleason grade (Collins et al. 2001; Collins et al. 2005). Many studies have reported that PCSCs are responsible for the initiation and maintenance of prostate cancer and metastasis. Prostate cancer likely initiates from luminal stem cell, but also from basal stem cells, which in turn evolve into luminal-like stem cells (Goldstein et al. 2010; Qin et al. 2012). Additional prostate cancer stem cell markers are integrin subunit $\alpha 6$, c-Met receptor and aldehyde dehydrogenase 1. Population of prostate cancer cells with low PSA levels has been found to be resistant to chemotherapy when compared with high PSA levels. PCSC cells are believed to be the driving force behind the cell population with low PSA. The low PSA expressing cells had overabundance of the stem cells markers $\alpha 2$, CD44 and aldehyde dehydrogenase 1, and had asymmetric cell divisions. The cells had also low or no levels of AR and produced larger tumors *in vivo* than cells with high PSA levels. (Qin et al. 2012). Primary prostate cancer tissues enriched with $\alpha 2$, $\alpha 6$ and c-Met (> 5% of the cells) mediate also significantly the progression of bone metastases (Colombel et al. 2012).

As in many cancer types, αV -integrins are also known to promote prostate cancer progression, especially during angiogenesis and formation of metastases in bone. Integrin $\alpha V\beta 3$ is essential in the formation of bone metastases, where it adheres to osteopontin and vimentin in the bone matrix, and activates the P13K/AKT pathway (Cooper, Chay, and Pienta 2002). Integrin $\alpha V\beta 6$ promotes osteolytic activity for formation of bone metastases by upregulating specifically MMP-2 (Dutta et al. 2014). Integrin $\alpha V\beta 5$ is expressed in the tumor and stromal cells and the expression correlates significantly with Gleason pattern and survival (Hess et al. 2014). Recently it has been reported that integrin $\alpha V\beta 6$, which is absent from normal prostate tissue, is enhanced in primary prostate cancer by exosomes, which are transported from $\alpha V\beta 6$ - positive cells to $\alpha V\beta 6$ -negative cells (Fedele et al. 2015).

2.3.4 Integrins in melanoma

The majority of the melanoma studies which concern collagen binding integrins have so far mostly concentrated on $\alpha 2\beta 1$. However, both $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are usually present in melanoma and have been found in primary and metastatic tissues (Klein et al. 1991; Schadendorf et al. 1993; Danen et al. 1994). In normal melanocytes, a study has shown presence of $\alpha 2$ where it is

mainly localized in the dendritic outgrowth (Hara et al. 1994). Integrins $\alpha V\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 1$ have been shown to be expressed in normal melanocytes (Kuphal, Bauer, and Bosserhoff 2005).

Studies have reported that $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are able to promote the migration and angiogenesis process in melanoma (Brown et al. 2008; Staniszewska et al. 2009). The binding of collagen IV to $\alpha 1$ and $\alpha 2$ seems to be critical for invasion and angiogenesis in melanoma (Pasco et al. 2005). Cyclic peptide sequences against $\alpha 1$ and $\alpha 2$ inhibited angiogenesis and decreased the proliferation of endothelial cells in a membrane assay (Momic et al. 2014). An *in vitro* study reported that $\alpha 2\beta 1$ is particularly important in melanoma growth and aggressiveness. Compared to other $\beta 1$ - integrins, $\alpha 2\beta 1$ was significantly expressed in primary cells derived from vertical growth phase and in metastases than in the primary cells from radial growth phase (Etoh, Byers, and Mihm 1992). *In vitro* and *in vivo* studies have detected enhanced levels of $\alpha 2\beta 1$ in highly aggressive melanoma types, where $\alpha 2\beta 1$ adheres specifically to collagen type I, IV and laminin (Danen et al. 1993; Klein et al. 1991). Integrin $\alpha 2\beta 1$ enhances the migration rate and invasion in high metastatic melanoma cell lines, particularly in the absence of the known pro-metastatic receptor integrin $\alpha V\beta 3$ (Etoh et al. 1993; Staniszewska et al. 2009). Lumican, which is an ECM protein in the skin and cartilage, decreases cell migration in melanoma *in vitro* by direct targeting of $\alpha 2$ (Zeltz et al. 2010). No correlation has been found between the expression of $\alpha 1\beta 1$ or $\alpha 2\beta 1$ and different melanoma types (superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma). No correlation has either been drawn between $\alpha 1\beta 1/\alpha 2\beta 1$ and ulceration (Schadendorf et al. 1993). One microarray study has found downregulated $\alpha 10$ mRNA in melanoma (Engel et al. 2013). Integrin $\alpha 11\beta 1$ has not been studied in either melanoma or normal melanocytes.

Integrin $\alpha V\beta 3$ is strongly connected with melanoma and is associated with invasiveness and melanoma tumor thickness (Hsu et al. 1998). Integrin $\alpha V\beta 3$ correlates with MMP-2 and matrix degradation. MMP-2, which is localized on the edge of the spreading melanoma cells, cleaves fibronectin and promotes the direction of the adhering and migrating $\alpha V\beta 3$ -mediated melanoma cells (Hofmann et al. 2000; Jiao et al. 2012). Overexpression of $\beta 3$ in primary melanoma cells promotes the switch from radial growth phase to vertical (epidermis into the dermis) in 3D skin reconstructs, inhibits apoptosis of invading cells and enhances tumor growth *in vivo* (Hsu et al. 1998).

2.3.5 Integrins in cancer therapy

Because integrins have a crucial role in cell migration, tissue invasion and angiogenesis, integrins are attractive targets in studies of cancer therapy. Currently, several integrin antagonists are being investigated for cancer treatment. These anti-cancer drugs block the endothelial-linked integrins $\alpha V\beta 3$, $\alpha V\beta 5$ and $\alpha 5\beta 1$, and are studied in clinical phases I-III trials. The drugs are produced as monoclonal antibodies or peptide antagonists, to suppress tumor growth, invasion and angiogenesis. Intetumumab, which targets αV has recently been evaluated in phase II studies for treatment of metastatic melanoma and metastatic CRPC, but unfortunately the studies led to non-significant results and lower survival outcome in patients, respectively (Heidenreich et al. 2013; O'Day et al. 2011). The anti-angiogenic therapy with endostatin (endostar, YH-16), which interacts with integrin αV and $\alpha 5$ on the endothelial cells, has been

approved in 2005 by China's State Food and Drug Administration (SFDA) for treatment of NSCLC (Wang et al. 2005; Rong et al. 2012). However, no anti-cancer drugs targeting collagen binding integrins have been in any clinical trials yet. Further studies are therefore needed to investigate the role of the collagen binding integrins in cancer. However, integrin α 1- (SAN-300) and α 2- (vatelizumab) antibodies are currently being evaluated in phase II trials for rheumatoid arthritis and relapsing remitting multiple sclerosis, respectively (www.clinicaltrials.gov).

2.4 CANCER TESTIS ANTIGENS

Cancer Testis antigens (CT antigens) are a group of tumor antigens and were the first human tumor antigens shown to be recognized by cytotoxic T lymphocytes (CTL) using T-cell epitope cloning (van der Bruggen et al. 1991). CT antigens are known to have a restricted expression pattern. As their name implies Cancer/Testis antigens are usually expressed in a range of cancer types, but are strictly restricted to the testis under normal conditions. Their expression may also be detected in the trophoblasts of the placenta and in ovarian germ cells. Under normal conditions, CT antigens have a role in gametogenesis (Simpson et al. 2005).

CT antigens are immunogenic proteins which mean that they are able to trigger an immune response. They are recognized by CTLs through the antigen peptides (epitopes) presented by the major histocompatibility complex (MHC) molecules I and II (or HLA) on the cell surface (Caballero and Chen 2009). However, normal tissues that express CT antigens (testis and placenta), are classified as immune privileged tissues, implicating that CT antigens are unrecognizable to the immune system in these tissues. Immune privileged tissues usually lack T-cells (due to absence of MHCs) and a tightly regulated form of immune control, which is not completely understood (Fiszer and Kurpisz 1998; Meinhardt and Hedger 2011). The immune privileged status of the testis is believed to be partly established by the tight blood-testis barrier, formed by Sertoli cells, which prevents the invasion of T-cells and thereby antigen recognition (Bart et al. 2002).

As CT antigens are tumor specific and are stably expressed and also have immunogenic features, they are considered ideal targets for cancer immunotherapy. They are also attractive candidates for diagnostic and prognostic biomarkers. At the moment, CT antigens are very attractive targets in vaccination trials. CT antigen based vaccines are used to trigger a reactive immune response, where CTLs (CD8⁺ T-cell) will recognize and destroy the CT antigen-positive tumor cells. For instance, NY-ESO-1 peptide vaccines have been able to induce CD4⁺ and CD8⁺ T-cells and antibody responses specific for several epitopes of the NY-ESO-1 antigens in cancer patients (Jager et al. 2006). Since CT antigens are also highly tumor specific, a CT antigen-based treatment is not expected to cause harmful autoimmune reaction as other immunotherapies (Srinivasan and Wolchok 2004; Suri et al. 2012). Several clinical trials have reported good tolerance to the CT antigen based vaccinations in the patients (Adam, Wauters, and Vansteenkiste 2014; Wada et al. 2014).

2.4.1 The families of Cancer Testis antigens

According to the CT database of the Ludwig Institute for Cancer Research (www.cta.lncc.br), 243 CT antigens have been discovered (April 2015), characterized and grouped into over 60 CT antigen families. The first cancer testis antigen, MAGE-1 (melanoma associated antigen, MAGE-A1), was discovered in 1991 in a screen for T-cell recognition sites in human melanoma cells (van der Bruggen et al. 1991). Even though the first CT antigen was found in 1991, the name Cancer Testis antigens (CT antigens or CTA) was not used until 1998, when a nomenclature for Cancer Testis antigen was created (Old and Chen 1998). A good system was needed for the expanding amount of known CT antigens. The nomenclature is based on the chronological order of the discovery or classification of a protein as a CT antigen, meaning that the first discovered CT antigen MAGE-1 corresponds to CT1. In the beginning of the 1990's new CT antigens were discovered with T-cell epitope cloning, but today they are mainly discovered by database mining or SEREX (Serological analysis of expression cDNA libraries) (Simpson et al. 2005; Chen, Gure, and Scanlan 2005). Overall, many CT antigens have been identified, but more effort is needed to characterize the discovered CT antigens.

2.4.1.1 CT-X antigens and non-X CT antigens

CT antigens can be divided into two groups based on their chromosomal location. One group, called the CT-X antigens, is located on the X-chromosome, and the other group, the non-X CT antigens, is distributed over various autosomes. The CT-X antigens make up over 50 % of all CT antigens. CT16, studied in this thesis, is classified as a CT-X antigen. The CT-X antigens are often organized into well-defined clusters and form expanded gene families on the X-chromosome (Simpson et al 2005). One cluster is located in the chromosome Xp11 region, which contains the GAGE/PAGE/XAGE super-family and includes CT16. The non-X CT antigens are often single copy DNA genes distributed along the entire genome (Simpson et al 2005).

MAGE (melanoma associated antigens) is the most studied and largest CT-X antigen family and is divided into three subfamilies: MAGE-A, MAGE-B and MAGE-C. MAGE, as the name implies, is expressed in melanoma at high frequency, but is also one of the most common CT-X antigens seen in other cancer types. As much as 70 % of metastatic melanoma tissues express either MAGE-A1 or MAGE-A4 (Brasseur et al. 1995). Other CT-X families are for example NY-ESO-1 (New York esophageal squamous cell carcinoma-1), GAGE (G antigen), BAGE (B melanoma antigen), XAGE (X antigen), SPANX (Sperm protein associated with the nucleus on the X chromosome), SSX (Synovial sarcoma X breakpoint) and PAGE (Prostate-associated gene) (CT database).

2.4.2 Cancer Testis antigen 16

Cancer testis antigen 16 (CT16) is a small protein containing 110 amino acids and is located on the X-chromosome (Xp11.21) (CT database; <http://www.cta.lncc.br>). Other synonyms for CT16 are PAGE-5, CT16.1 or GAGEE1. CT16 has two splice variants. Variant 1 (NM_130467, CT16.1) has a longer segment of nucleotides in its N-terminal region compared to variant 2

(NM_001013435, CT16.2). CT16 belongs to the prostate associated antigen family: PAGE, because of the sequence identity it shares with other members of the PAGE family. The PAGE family has six members consisting of PAGE-1, PAGE-2, PAGE-2B, PAGE-3, PAGE-4 and PAGE-5 (CT16). CT16 was identified in 2002 by database mining from normal testis and tumor cDNA libraries (Scanlan et al. 2002a). CT16 shares a sequence identity of 30-50 % with the CT antigens of the GAGE family (Scanlan et al. 2002a).

Only a few studies on CT16 have been published. One study has observed detection of CT16 in human tissues from melanoma (4 out of 18), lung cancer (7/18) and renal cancer (7/16) and in one case of colon (1/9) and breast cancer (1/18) by qRT-PCR (Scanlan et al. 2002a). In the same study, detection of CT16 was also reported in some cell lines from melanoma, lung cancer and synovial cell carcinoma. However, no specific amount or concentration of CT16 was measured, only positivity. No CT16 was detected in normal melanocytes (Scanlan et al. 2002a). CT16 expression at protein levels has not been investigated in any tissue or cell line. Additionally, no CT16 studies have been done in prostate cancer, even though CT16 belongs to the PAGE family. CT16 has a high sequence identity with other PAGE members and belongs therefore to the PAGE family.

According to one *in silico* gene expression analysis of all CT antigens, CT16 belongs to the category of testis-selective CT antigens and not to the testis-specific antigens or testis/brain restricted antigens. This is due to positive detection, but very weak findings, in normal liver (Hofmann et al. 2008). So far, the biological function of CT16 is completely unknown. CT16 is also a highly intrinsically disordered protein (IDP) (lack of ordered 3D structure protein) (Hellman et al. 2011). IDPs can in general be totally unstructured or partially structured with some random coils. According to *in silico* prediction methods, over 90 % of CT antigens seem to be IDPs. When CT-X antigens were compared with non-X CT antigens, CT-X antigens were significantly more likely to be disordered (Rajagopalan et al. 2011). Despite the lack of structure, some IDPs have the ability to interact with partners like DNA through high-specificity/ low-affinity interactions and are important in signaling and transcriptional regulations (Vuzman and Levy 2012; Wright and Dyson 2015). IDPs are frequently associated with diseases such as cancer and cardiovascular diseases (Uversky et al. 2014).

2.4.2.1 Other members of PAGE- family

The first PAGE family member, PAGE-1, was discovered in hybridization screening of cDNA library from the metastatic prostate cancer LnCap subline (Chen et al. 1998). After the characterization of PAGE-1, five other members have been included in the family: PAGE-2, PAGE-2B, PAGE-3, PAGE-4 and PAGE-5 (Brinkmann et al. 1998; Iavarone et al. 2002; Scanlan et al. 2002a).

The most studied PAGE member is PAGE-4. It was identified by database mining and has a sequence identity of 43 % to CT16 (Brinkmann et al. 1998). PAGE-4 is a cytoplasmic protein and highly expressed in symptomatic BPH and in primary prostate cancer, but is reduced in prostate metastases and CRPC (Iavarone et al. 2002; Suyama et al. 2010; Zeng et al. 2013). PAGE-4 is also found in endometrial cancer, cervical cancer and colorectal cancer (Iavarone et al. 2002; Chen

et al. 2010). PAGE-4 has possibly a role in the embryonic development. In normal tissues, PAGE-4 is expressed at moderate levels in the prostate and uterus, but is highly expressed in the testis, fetal prostate, fetal testis and placenta (Iavarone et al. 2002; Zeng et al. 2013).

Recently, it was found that PAGE-4 acts as a critical member of the stress-response pathway, which plays an important role in the development of prostate cancer. Elevated levels of PAGE-4 have been measured in prostate cancer cell lines treated with stress factors such as TNF- α and glucose deprivation. PAGE-4 is able to transactivate the proto-oncogene c-Jun, which is known to get activated by the stress-response kinase JNK-1 (Rajagopalan et al. 2014). Since stress response function has critical influences on apoptosis, PAGE-4 seems to contribute to the development of prostate cancer through an anti-apoptotic role as well. Elevated levels of p21, phosphorylated p53 and the double strand breakage marker γ H2A.X have been observed in prostate cancer cells after PAGE-4 knock down. PAGE-4 siRNA has decreased tumor growth in LnCap xenografts (Zeng et al. 2011).

PAGE-4 appears also as a good indicator of developing liver metastases in colorectal cancer. When PAGE-4 was included in a panel model of two other risk factors (vessel cancer embolus and number of lymph nodes), the prediction risk of developing metastases was improved with a unit of almost 20 %. The predicted risk was consistent with the actual risk. The metastases prediction with only two risk factors; lymph nodes ($n \geq 4$) and vessel cancer embolus, was 57.5 %, and when PAGE4 was included the risk became 77.2 %. The frequency of PAGE4 mRNA expression was significantly higher in primary colorectal tumors with liver metastases compared to tumors without metastases, 39 % versus 21 %, respectively (Chen et al. 2010).

2.4.3 Regulation of Cancer Testis antigen-expression

The expressions of CT antigens have been shown to be regulated by epigenetic factors, and DNA methylation is suggested to be the primary factor (Fratta et al. 2011). DNA methylation is a silencing mechanism of gene transcription and it refers to addition of a methyl group to a cytosine residue within cytosine-guanine rich regions (CpG islands). DNA methylation is associated with processes like genomic imprinting and X-chromosome inactivation (De Smet et al. 1999; Robertson and Jones 2000). A disruption of DNA methylation occurs in malignancies.

Weber et al made the first observation that DNA methylation was involved in the regulation of CT antigens. They showed that the demethylating agent 5-aza-2-deoxycytidine (AZA) upregulated MAGE-A1 expression in melanoma *in vitro* (Weber et al. 1994). Further on, transfected methylated and demethylated promoter plasmids in metastatic melanoma cells have shown to regulate the production of MAGE-2, -3 and -4 (Sigalotti et al. 2002). The transcription of CT-X antigens seems to be more regulated by DNA methylation than non-X CT antigens (Yao et al. 2014). A microarray analysis in prostate cancer cells has shown that about 18 % of all demethylated genes in the genome were CT-X antigens. DNA demethylation was mostly pronounced in metastases than in primary tumors of prostate cancer, suggesting that demethylation in prostate cancer participates mostly in formation of metastases rather than in cancer initiation (Yegnasubramanian et al. 2008). Histone modifications, such as histone acetylation and methylation, are other epigenetic factors, which participate in the regulation of CT antigens (Wischniewski, Pantel, and Schwarzenbach 2006). Histone methylation is thought to

be involved in the regulation of the NY-ESO-1, MAGE-A1 and MAGE-A3 antigens in lung cancer (Rao et al. 2011). Histone acetylation and methylation might also play important assisting roles in DNA methylation. In breast cancer GAGE antigens can be regulated by all three mechanisms, since they were induced most optimally by a combination of AZA, the histone methylation inhibitor DZNep (3-Deazaneplanocin) and the histone deacetylase inhibitor TSA (trichostatin) (Sun et al. 2009). The possibility of combining CT antigen immunotherapy with epigenetic drugs has been suggested. Apart from DNA methylation and histone modifications, tissue or subtype-specific transcription factors also contribute to the regulation of CT antigen-expression (Yao et al. 2014).

2.4.4 Cancer Testis antigens in normal tissues

CT antigens in normal tissues are predominantly expressed in human germ cells in testis, but are also found in the ovaries, placenta, fetal testis, fetal ovaries and embryonic stem cells (Satie et al. 2002; Nelson et al. 2007; Lifantseva et al. 2011). In normal tissues, CT antigens participate in spermatogenesis and oogenesis, collectively called gametogenesis. CT-X-positive germ cells appear as early as 13 weeks after gestation (Chen et al. 2011). CT antigens are absent in normal somatic cells. However, variable minor (< 1 %) mRNA expression of CT antigens in the pancreas, liver, kidney, spleen, and thyroid gland has been detected, but the biological significance of this expression is considered to be low (Scanlan, Simpson, and Old 2004). Additionally, no CT protein expression in these tissues has been confirmed (Scanlan, Simpson, and Old 2004; Caballero and Chen 2009).

Non-X CT antigens are better characterized in normal testis than CT-X antigens. CT-X antigens usually participate in the initial stages of spermatogenesis, whereas non-X CT antigens are frequently expressed in later stages. CT-X antigens are predominantly detected in spermatogonial stem cells and in primary spermatocytes (Satie et al. 2002). Non-X CT antigens have significant roles in meiosis, sperm metabolism and movement, which contribute to the effects of fertility (Caballero and Chen 2009; Whitehurst 2014).

2.4.5 Cancer Testis antigens in cancer

CT antigens are heterogeneous in their expression patterns, which vary among different cancer types and tumor grades. Some carcinomas are classified as CT antigen-rich, while others are CT antigen-poor (Scanlan, Simpson, and Old 2004). Melanoma, lung cancer, bladder and ovarian cancer are often CT antigen-rich. In this case, CT antigen rich refers to that around 50 % of the samples in a tumor collection express the same CT antigen (Scanlan, Simpson, and Old 2004). An intermediate frequency of CT antigens seems to be typical for breast and prostate cancer (Caballero and Chen 2009). A low frequency is usually seen in leukemia, Hodgkin's and non-Hodgkin's lymphomas, glioblastoma, renal and colon carcinoma (Caballero and Chen 2009).

Some CT antigens are very frequently expressed. MAGE-A1, MAGE-A3, NY-ESO-1, SSX-2, SSX-4, PLAC1 and PRAME appear to be the most common CT antigens (Scanlan et al. 2002b; Yao et al. 2014). It is suggested that CT antigens are expressed in a coordinated pattern (Gure et al. 2005;

Barrow et al. 2006). If one CT antigen is expressed the probability that another CT antigen is present increases 1.5 to 3 times (Gure et al. 2005). In one co-expression study, where nine CT-X antigens were investigated, 26% of the melanoma tumors expressed none of the nine CT-X antigens whereas 65% of the melanoma tumors expressed three or more of the CT-X antigens (Sahin et al. 1998). CT-X genes, which are located closely in the same chromosomal cluster, are often co-expressed. The MAGE-A genes and the CSAG genes, which are only 70 kb apart at Xq28, are frequently activated together (Yao et al. 2014). Significant correlations between the co-expression of MAGE-A10 and NY-ESO-1 and topoisomerase 2-alpha (TOPO2A) have been observed in triple negative breast cancer (Mrklic et al. 2014).

2.4.5.1 Cancer Testis antigens in cancer therapy

Today, CT antigen vaccines are being tested in numerous clinical trials (<https://clinicaltrials.gov>, <https://www.clinicaltrialsregister.eu>). The vaccines which are protein or peptide based vaccines are designed to boost CTLs (CD8⁺ T-cells) in order to recognize CT antigen-positive cancer cells by MHCs. By targeting the cancer cell the CTLs is aimed to destroy the cell and decrease the tumor burden (Jager, Jager, and Knuth 1999; Comber and Philip 2014). MAGE-A3 and NY-ESO-1 are the best characterized and most used CT antigens in clinical trials (Comber and Philip 2014). They are being investigated for their potential for the treatment of several cancer types, including advanced melanoma, lung cancer and prostate cancer. The therapy has so far been shown to promote immune responses, but with controversial clinical success. Trials with a combination of CT antigen vaccines and CTLA-4 inhibitors are on-going in melanoma (clinicaltrials.gov). Multi-CT antigen therapy, based on the prioritized co-expression of CT antigens, has also been put into focus for increasing the immune response (Okuyama et al. 2013).

2.4.5.2 Expression of Cancer Testis antigens during cancer progression

CT antigens are generally expressed at higher frequencies in higher graded primary tumors and metastasis compared to lower graded tumors. NY-ESO-1 correlates with higher tumor grades in NSCLC, bladder cancer and melanoma (Konishi et al. 2004; Kurashige et al. 2001). In melanoma, NY-ESO-1 had an mRNA expression of 3.5 %, 9.5 % and 45.5% in grade I, II and III melanomas, respectively. Primary NY-ESO-1 melanomas were also notably thicker (4.7 mm) than negative NY-ESO-1 melanomas (1.5 mm) (Velazquez et al. 2007).

Poor prognoses for cancer recurrence and shorter survival have been connected to the presence of CT antigens (Shiraishi et al. 2011; Shigematsu et al. 2010; Riener et al. 2009). However, little is known about this prognosis correlation, partly due to few studies and small malignancy collections. One large melanoma tissue study ($n=348$) has demonstrated that MAGE-A1, MAGE-A4 and NY-ESO-1 may act as strong predictors of a shorter relapse free survival (RFS) in primary cutaneous melanoma (stage I and II) (Svobodova et al. 2011). The RFS was 45 months in MAGE-A1, MAGE-A4 and NY-ESO-1 positive tumors versus 72 months in tumor negative for these markers. The expression levels of MAGE-A1, MAGE-A4 and NY-ESO-1 were significantly co-expressed with each other and higher expression of them were associated with increased tumor

thickness, presence of ulceration, female gender and increased age at diagnosis. However, co-expression of MAGE-A1, MAGE-A4 and NY-ESO-1 did not have an influence on overall survival and was not associated with the anatomic tumor site, melanoma subtype or tumor infiltrating lymphocytes (Svobodova et al. 2011). MAGE-C2 has been shown to be more frequently expressed in prostate cancer metastases and CRPC compared to primaries and may also function as an independent predictor of recurrence in prostate cancer after radical prostatectomy (von Boehmer et al. 2011).

2.4.5.3 Function of Cancer Testis antigens in cancer

Research on the roles of CT antigens during cancer progression has mostly focused on the MAGE and SSX CT antigen families. Studies have also been carried out with GAGE, PAGE-4, IL13RA1 and HCA661. MAGE antigens, which are associated with aggressive tumor growth, poor clinical outcome and chemotherapy resistance, are known to promote tumor cell survival by repressing the activity of p53 (Bhatia et al. 2013; Monte et al. 2006). The MAGE antigens form a complex with the RING domain protein TRIM28 (Tripartite motif-containing 28)/KAP-1 (KRAB associated protein-1), which carries an E3 ubiquitin ligase activity to promote the polyubiquitination and proteasomal degradation of p53 (Yang et al. 2007; Doyle et al. 2010). MAGE-A also inhibits apoptosis by p53-independent mechanisms, by enhancing the expression of the anti-apoptotic gene survivin (BIRC5) in myeloma cells (Nardiello et al. 2011). Survivin is a target gene of the Wnt pathway, and acts as a direct inhibitor of caspase-3 and -7 (Shin et al. 2001; Olie et al. 2000). Increased levels of survivin correlate with tumor growth and poorer prognosis in primary multiple myeloma (Romagnoli et al. 2009). MAGE-C2 enhances the melanoma cell growth through phosphorylation of KAP-1 at Ser824 and by increasing the DNA damage repair (Bhatia et al. 2013).

The CT antigen SSX (Synovial Sarcoma X) appears to be a transcriptional regulator with roles in cell proliferation, survival and EMT (D'Arcy et al. 2014). CT antigen SSX is expressed abundantly in melanoma and promotes the cell proliferation and survival in melanoma through the MAPK/ERK signaling pathway. SSX-siRNAs could also decrease the growth of melanoma tumor xenografts (D'Arcy et al. 2014). SSX can also interact and form a complex with β -catenin to activate the transcription of EMT associated genes: E-cadherin, snail-2 and vimentin. The dysregulation of Wnt/ β -catenin may lead to numerous malignancies (D'Arcy et al. 2014; Fodde and Brabletz 2007).

The cancer type synovial cell sarcoma is triggered by chromosomal translocations (breakpoints) between the SYT gene (Synovial sarcoma Translocated gene) on chromosome 18, and the SSX antigen on X chromosome 11, causing a SYT-SSX fusion gene. The fusion gene both represses and activates transcription of its target genes. Through DNA binding proteins, it seems to directly repress the cell proliferation regulator COM1 (candidate of metastasis-1), which leads to enhanced cell growth and decreased apoptosis (Ishida et al. 2007). SYT-SSX triggers the progression of synovial sarcoma through the ERK pathway by inducing the expression of ERK1/2, p-ERK, cyclin D1 and CDK4 (Cai et al. 2011).

2.4.6 Cancer Testis antigens in prostate cancer

The knowledge about CT antigens in prostate cancer is fairly limited. By comparing several studies, which investigated the expression frequencies of CT antigens in many cancer types, Scanlan et al. divided cancer types into three groups: high, moderate and low CT antigen expressers. Prostate cancer belonged to the moderate CT antigen expressers, since six out of the twenty investigated CT antigens had a frequency of > 20 % in prostate cancer samples (Scanlan, Simpson, and Old 2004). The six CT antigens were SSX-2 (Tureci et al. 1998), NY-ESO-1 (Chen et al. 1997), HAGE (Martelange et al. 2000), LDHC, TPX1 (Koslowski et al. 2002) and TDRD1 (Loriot, Boon, and De Smet 2003). SSX-2 had the highest frequency with 40 %, followed by TDRD1 with 38 %, whereas HAGE had the lowest frequency with 22 %. Of all the nine members in the SSX family, SSX-2 seems to be the main CT antigen member in prostate cancer (Smith et al. 2011; Dubovsky and McNeel 2007). However, SSX-2 has mostly been found in metastatic prostate tissues compared to primary lesions. The expression of SSX-2 is regulated by DNA methylation (Dubovsky and McNeel 2007; Smith et al. 2011). SSX-2 is also a potential target for metastatic prostate cancer vaccine therapy. Plasmid DNA vaccines in mice have been able to trigger a response of epitope-specific CD8⁺ T cells targeting SSX-2 (Smith and McNeel 2011).

Of the members of the PAGE family, PAGE-1 and PAGE-4 have been associated with prostate cancer so far. The expression of PAGE-2, PAGE-2B and PAGE-3 are still uninvestigated (CT database). PAGE-1 is suggested to enhance prostate cancer progression. A poor expression of PAGE-1 mRNA has been scored in the non-tumorigenic LNCap cell line (androgen dependent), but was increased five-fold in the metastatic and androgen independent mice sublines of LNCap. However, the overexpression was not regulated by androgens since no difference was seen by androgen treatment with dihydrotestosterone (Chen et al. 1998). Another study reported that no PAGE-1 mRNA was detected in human LNCap cell lines or human prostate tumor tissues, which included five primary and two metastatic tumors (Dannull et al. 2000).

PAGE-4 has been detected in prostate cancer at both the mRNA and protein level (Brinkmann et al. 1998; Iavarone et al. 2002). A microarray study reported that PAGE-4 is expressed at higher levels in benign and primary prostate cancer tissues than in metastatic tissues (Suyama et al. 2010). In the same study, the expression patterns of the CT antigens MAGE-A2, MAGE-A12, SSX-2 and CSAG2 were the opposite. They were more upregulated in metastases compared to benign and primary prostate cancer (Suyama et al. 2010). PAGE-4 is a stress-response protein and it regulates c-Jun transactivation in prostate cancer (Mooney et al. 2014).

2.4.7 Cancer Testis antigens in melanoma

MAGE is clearly one of the most frequently expressed CT antigen families in melanoma. The MAGE-A subfamily is known as the best characterized family. Over 70 % of melanoma tissues express at least one member: MAGE-A1, MAGE-A2, MAGE-A3 or MAGE-A4 (Brasseur et al. 1995). In cutaneous melanoma, the mRNA expression of these antigens correlates significantly with tumor thickness and ulceration. Their expression frequency is highly increased in metastases compared to primary tumors (Brasseur et al. 1995). No expression correlations have been made between different melanoma metastases. Benign nevi, dysplastic nevi and

cutaneous *in situ* melanomas do not express MAGE antigens (Brasseur et al. 1995; Basarab et al. 1999). Further, ocular melanomas seem to express low levels of MAGE antigens (Errington et al. 2012).

MAGE-A3 is the knock out target in an extensive phase III melanoma adjuvant vaccination trial (DERMA), involving 1300 MAGE-A3 positive cutaneous melanoma patients worldwide (www.clinicaltrialsregister.eu). The aim is to prevent the recurrence of melanoma tumors after surgery in patients with stage IIIB/C tumor. The first primary endpoint (2013) of the trial did not extend the disease-free survival. However, the trial continues in order to find a gene signature that could identify a sub-population of the melanoma patients that were benefitted of the vaccine (Ascierto et al. 2015). MAGE-A3 has been identified as a frequent CT-antigen in melanoma metastases in skin (63% frequency), lymph nodes (65%) and internal organs, mostly liver (53%). The frequency was independent of age, gender, duration of the disease (from primary tumor to appearance of metastases) or of the histological type of the primary melanoma (Roeder et al. 2005). In addition to MAGE-A3, qRT-PCR studies have also reported very high frequencies of MAGE-A6 (64-100 %) and MAGE-A12 (74%-83%) expression in melanoma lesions. Several epitopes of MAGE-A6 and MAGE-A12 have been identified to evaluate the immunogenicity and it has been suggested that they could be included into cancer vaccination trials (Breckpot et al. 2004; Akiyama et al. 2012; Gibbs et al. 2000).

Overall, melanoma is classified as a cancer type which generally expresses various CT antigens. Melanoma has been shown to express at least 17 different CT antigens, which have an expression frequency of over 20 %, including MAGE, NY-ESO-1, SSX-2, GAGE and BAGE-1 (Scanlan, Simpson, and Old 2004). Apart from MAGE, CT antigen NY-ESO-1 is another prioritized drug vaccination target in melanoma due to its good immunogenicity and high mRNA and protein frequency in metastases (Goydos, Patel, and Shih 2001; Velazquez et al. 2007; Aung et al. 2014). Correlations between tumor thickness and NY-ESO-1 expression have also been confirmed in melanoma primary tumors. NY-ESO-1 positive primaries were significantly thicker than NY-ESO-1 negative primary tumors. However, no significant differences in age, gender or presence of infiltrated lymphocytes were found between the NY-ESO-1 positive and NY-ESO-1 negative primaries (Velazquez et al. 2007). The presence of T-cells targeting NY-ESO-1 positive tumor cells is significantly associated with increased median overall survival in metastatic melanoma patients. The median overall survival was 21 months in patients with T-cells targeting NY-ESO-1 *versus* 6 months in patients without NY-ESO-1 T-cells (Weide et al. 2012). However, not all studies have documented an increase of NY-ESO-1 during the progression of melanoma (Barrow et al. 2006). Barrow and colleagues reported a complete loss of NY-ESO-1 in serial biopsies from the same patients, which suggest that NY-ESO-1 may take part in “immunoediting” during cancer progression.

3. AIMS OF THE STUDY

1. To analyse the mRNA expression of collagen binding integrins in various human sarcoma-, carcinoma- and melanoma cell lines.
2. To analyse the expression of collagen binding integrins in a large clinical collection of human localized prostatic carcinoma samples.
3. To study the impact of $\alpha 1$, $\alpha 2$ and $\alpha 11$ on survival in metastatic melanoma patients. The mRNA expression was measured in tissue samples obtained from metastatic melanoma patients.
4. To investigate the presence of CT16 in prostate cancer and metastatic melanoma by analysing the mRNA and protein expression of CT16.
5. To study the diagnostic and prognostic significance of CT16 as well as to investigate the biological role of CT16.

4. MATERIALS AND METHODS

4.1 Cancer cell lines (I,III, IV) and stable cell lines (IV)

The human cancer cell lines were obtained originally from American Type Culture Collection (ATCC, Rockville, MD). A collection of 24 cell lines (3 melanomas, 5 sarcomas and 16 carcinomas) were used in paper I to investigate the mRNA expression pattern *in vitro* of collagen binding integrins in sarcomas, carcinomas and melanomas. The cell lines from all studies (I, III, IV) were cultured at 37°C with 5 % CO₂ in DMEM + 10 % fetal calf serum (FCS), except cancer cell lines: T47D, NCI-H520, NCI-H2170, NCI-H1563, NCI-H2228, LNCap, Tera-1 and Tera-2, which were maintained in RPMI + 10 % FCS. The melanoma cell lines, A2058, SK-Mel-2 and WM-266-4, which have been mostly used in paper III and IV were authenticated and quality controlled by Health Protection Agency Culture Collection (Porton Down, UK). WM-266-4 cells were transfected with 1 µg of a CT16 cDNA-containing or empty (negative control) pEXPR-IBA103 vector with Fugene 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. The G418 resistant cells were selected with 0.8mg/ml of G418 (Geneticin) and the resistance was maintained with 0.2 mg/ml of G418.

4.2 Tissue and serum samples

4.2.1 Prostate cancer tissue samples (I, III)

The prostate cancer tissue specimens were obtained from 104 prostate cancer patients at the Turku University Central Hospital during the years 1999-2002. Two samples were taken from each prostate tissue, one from each side during a control palpation. Samples for RNA isolation were stored at -80°C in a solution containing 4 M guanidium thiocyanate, 25 mM sodium citrate dehydrate, 0.5 % sodium lauryl sacrosinate and 0.7 % 2- mercaptoethanol. The samples were frozen for histological grading and IHC. The prostate tissue samples were graded according to the Gleason scale (I-V) or scored as benign or Low/High PIN (prostatic intraepithelial neoplasia). The study was approved by the Ethic Committee of University of Turku and the patients had provided a written consent to study their tissues samples for research purposes.

4.2.2 Advanced melanoma samples (II-IV)

The melanoma samples were obtained from 26 patients with advanced melanoma treated at the Helsinki University Hospital during the years 1988-1996. All patients were treated with a combination of chemotherapy with the drugs vincristine, bleomycin and lomustine together with natural leukocyte interferon- α (IFN- α). The tumors were localized in the subcutis ($n=12$), lymph nodes ($n=12$), soft tissue ($n=1$) and orbit ($n=1$). The median age of the patients was 51 years and 18 of all patients were men and 8 were women. The tumors were graded according to the classification system of the WHO. Twelve and fourteen tumor samples were graded as stage III and IV melanoma, respectively. One patient suffered from severe side effects. The

patient's chemotherapy was discontinued and the patient was withdrawn from the survival study after the initiation of therapy.

The human melanoma tissue samples were obtained during normal surgical procedures done for the diagnosis or treatment of metastatic melanoma. The removed samples were cut in half and one part was put into liquid nitrogen and stored at -80°C , while the other part was formalin-fixed for pathological diagnosis and IHC analysis. 50-150 mg of each sample was used for RNA isolation. The clinical study was approved by the local Ethics Committee and the patients gave a written informed consent to study their melanoma tissues for research purposes.

Integrin $\alpha 1$, $\alpha 2$ and $\alpha 11$ mRNA expression of the melanoma tissue samples was determined with qRT-PCR (chapter 4.5). Kaplan-Meier survival curves were used to analyse the survival time difference between two patient groups. The patient groups were 1) patients with high mRNA expression of $\alpha 1$ (alternatively $\alpha 2$ or $\alpha 11$) and 2) patients with low mRNA expression of $\alpha 1$ (alternatively $\alpha 2$ or $\alpha 11$).

4.2.3 Serum samples from advanced melanoma patients (III)

Serum samples were obtained from 23 patients with metastatic melanoma of cutaneous, uveal or unknown origin. All patients had stage IV metastatic melanoma. The samples were collected without additives between years 2006 - 2008 at the University Central Hospitals of Helsinki and Turku and stored at -70°C . Two negative control groups were used: A group of serum samples from nine healthy volunteers collected from Turku University Hospital and a control group with 20 commercial serum samples, which were reported to be healthy (Vitalprod, Boynton Beach, FL). CT16 was measured with a time-resolved fluoroimmunoassay (chapter 4.9) in all serum samples. The general melanoma biomarkers S100B and LDH were also analysed with fluoroimmunoassay.

CT16 and S100B concentration was further analysed in sera from four patients: Patient A, B, C and D. The sera follow-up samples from the four patients were collected during the melanoma treatment. The same serum follow-up samples from patient A, B and C had previously been investigated in a clinical phase II trial (Vihinen et al. 2010). A study containing the serum samples from patient D has also been published (Vihinen and Pyrhonen 2008, www.karger.com). Patient A, B and C received the same first-line treatment. The treatment consisted of dacarbazine and bevacizumab (anti-VEGF antibody) in a cycle of 28 days. Additionally, a daily dose of interferon (IFN)- $\alpha 2a$ was given subcutaneously starting on day 15. Patient D received four different treatments, but the analysis of CT16 started from the second-line treatment. The second-line treatment consisted of carboplatin, vinorelbine, interleukin (IL)-2 with a cycle of 21 days. IFN- $\alpha 2b$ was also given at days 2-5 and 9-12.

CT16 and S100B were measured during the first-line treatment until week 15, 68 and 29 for patients A, B and C, respectively. CT16 and S100B were measured during the treatments until week 26 for patient D. No further serum samples were available after these time points. The first sample of each follow-up collection was obtained before the treatment started. The clinical studies were approved by the local Ethics Committees. Each patient had provided written

informed consent to participate in the trials and to study their tissue or sera for other research purposes.

4.3 Antibody for CT16 (III, IV)

An antibody against CT16 was produced at Eurogentec (Liege, Belgium) by immunizing two rabbits with human recombinant CT16. The antibody was custom made by order since there was no commercial CT16 antibody available at that time. The polyclonal CT16 antibody was purified from rabbit serum by affinity chromatography with two different columns, HiTrap Protein G HP affinity column or HiTrap NHS-activated HP column (GE Healthcare).

4.4 Total RNA isolation and cDNA synthesis (I-IV)

Total RNA was isolated from cultured cell lines and melanoma tissue samples using Trizol (Invitrogen) according to the manufacturer's protocol. Total RNA from the prostate cancer samples was extracted with a modified guanidinium-thiocyanate-phenol-chloroform extraction method (Chirgwin et al. 1979; Aaltonen et al. 1999). The isolated RNA was washed with 75% ethanol and measured for concentration and purity. 1 µg of the RNA was transcribed to cDNA. RNA (1 µg) was treated with 5 U of RNase free Dnase I (Roche) to eliminate a possible DNA contamination. The RNA was reverse transcribed into cDNA using a dNTP mix, 200 U of M-MLV Reverse Transcriptase, 1 x M-MLV Reverse transcriptase buffer and 0.5 µg of random hexamer primers in a total volume of 25 µl (Promega, Madison, WI). The reverse transcription was carried out at 37°C for 1 hour.

4.5 Real Time quantitative Reverse Transcription PCR (I-IV)

Forward and reverse primers and probes for human α 1, α 2, α 10, α 11, androgen receptor, CT16, DKK-1, CTSL and β -actin were self-designed, using the Primer Express 3.0 software (Applied Biosystems). They were synthesized by Eurogentec (Belgium) or Oligomer (Finland). The probes were labeled at the 5'-end with the fluorophore 6-carboxyfluorescein (FAM) and at the 3' end with the quencher carboxy-tetramethyl-rhodamine (TAMRA). Primers and probes for human MT2A, DDAH1, BIRC5 and FABP7 were ordered as a gene expression assay mix (20x), consisting of forward and reverse primers and a 6-FAM/MGB labeled probe (Applied Biosystems, Waltham, USA). The sequences of the primers and probes are listed in a table (IV, Supplementary Table S2)

Real Time quantitative Reverse Transcription PCR (Real-Time qRT-PCR) was performed in 96-well plates. In a final volume of 10 µl, one well contained 5 µl of the Taqman Universal PCR Master mix (5x, Applied Biosystems), 2 µl of cDNA (diluted 1:10), 200 nM of probe, 300 nM of the forward primer and 300 nM of the reverse primer (self-designed primers and probes). When using the gene expression assay mix instead of self-designed primers and probes, 0.5 µl of the gene expression assay mix + 2,5 µl H₂O were added to one well. Three parallel qRT-PCR samples

were made from every cDNA. The qRT-PCR was run with an ABI PRISM 7700 or 7900HT Sequence Detection System (Taqman, Applied biosystems). The run was programmed with a 2 min initiation at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60 °C. A C_T (cycle threshold) value was determined for each sample. The C_T value is the cycle number (max 40) required for the fluorescent signal to reach the set threshold unit (in practice: 0.05). The C_T value of the test gene (for example $\alpha 1$) was normalized with the C_T value for the endogenous reference gene β -actin, (C_T (test gene) - C_T (β -actin)) = ΔC_T . The mean relative mRNA expression was calculated for each sample and presented as a percentage. This was done by using the formula $(2^{-\Delta C_T}) \times 100$.

4.6 RNA interference (IV)

RNA interference using CT16 siRNA1 and 2 (Eurogentec) was performed in the melanoma cell lines A2058 and SK-Mel-2 to knock down CT16 mRNA. Both CT16 siRNAs targeted both CT16 variants 1 and 2. A non-targeting siRNA was used as a negative siRNA control (Eurogentec). The specific sequences of CT16 siRNA1 and siRNA2 were 5'-GCUGGAAAUUUGACUGCUAdTdT-3' and 5'-GGGACUGAUGUGGAAGCUUdTdT, respectively. The sequence of the negative siRNA control was 5'-AAGAUUCGUAACCGUUGUGdTdT. The siRNA transfection was performed with the siLentFect Lipid reagent (Bio-rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. The siRNA transfected cells were incubated for 48, 72 or 96 hours. CT16 was inhibited in melanoma cell lines with 300 nM CT16 siRNA1 and 2 (IV, S 3A-C).

4.7 Western blotting (III-IV)

For Western Blotting (WB), total proteins were extracted from the cultured cancer cell lines with lysis buffer. The protein concentrations of the lysates were measured with the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were run on 12 % non-denaturing (CT16) or on 10 % - 12 % denaturing polyacrylamide gels (DKK-1, p53, Cleaved Caspase-3, MAGE-1, GAGE-1), and then transferred to a nitrocellulose membrane. To reduce background staining, the membrane was blocked for 1 hour with 5 % nonfat dry milk + 1 % BSA and 0.1% Tween. The membrane used for the detection of p53 was blocked in 5 % BSA + 0.1% Tween. The primary antibodies are listed in Table 3. The used secondary antibodies were HRP-conjugated anti-rabbit IgG (sc-2054 1:5000, Santa Cruz Biotechnology, Santa Cruz, CA), HRP-conjugated anti-mouse IgG (sc-2005, 1:5000, Santa Cruz Biotechnology) or HRP-conjugated anti-goat IgG (sc-2045, 1:5000, Santa Cruz Biotechnology). The SuperSignal West Pico (Thermo Fisher, Waltham, MA) was used as the chemiluminescent HRP substrate to detect the proteins. DKK-1 was detected as a secreted protein from the medium of the cultured cells. Before the gel run, proteins in 2 ml of medium were concentrated using a centricon centrifugal filter (YM-10, Millipore, Billerica, MA) by centrifuging at 5000 x g at 4°C, until the volume of the medium was approximately 60 μ l. The protein concentration was equally loaded to the gel.

Table 3. List of primary antibodies

Antigen	Primary antibody (clone/number)	Dilution	Producer
DKK-1	H-120/sc-25516	1:500 (WB)	Santa Cruz Biotechnology
Cleaved Caspase-3	polyclonal/9661 (Asp-175)	1:500 (WB)	Cell Signaling Technology
p53	DO-1/ sc-126	1:500 (WB)	Santa Cruz Biotechnology
MAGE-1	6C1/ab49386	1:250 (WB)	Thermo Fischer Scientific
GAGE-1	polyclonal/ab67412	1:500 (WB)	Abcam
α 1	CD49a/SR84	1:750 (IHC)	BD Pharmingen
α 2	MAB1950/P1E6	1:1000 (IHC)	Chemicon
AR	N-20	1:1000 (IHC)	Santa Cruz Biotechnology
Custom made antibodies			
α 11	polyclonal, IgG (Velling et al 1999)	1:500 (IHC)	Innovagen
CT16	polyclonal, IgG (Rappu et al 2011)	1:500 (WB)	Eurogentec
CT16	polyclonal, IgG (Rappu et al 2011)	1:10000 (IHC)	Eurogentec

4.8 Immunohistochemistry and Immunofluorescence (I, II and IV)

In paper I the protein expression levels of α 2, α 11 and the androgen receptor were investigated in prostate cancer by IHC. Prostate tissue samples, graded as benign, low PIN or Gleason grade 1-5, were included in the IHC study. The protein expression was done to compare it with the corresponding mRNA expressions, and to investigate the localization of α 2 and α 11. In paper IV, CT16 was stained by IHC in tissues of advanced melanoma, prostate cancer and normal testis tissue samples to localize CT16. In paper II, α 1 and α 2 were detected by IHC in melanoma samples.

Frozen prostate and melanoma sections were cut with a cryostat into 6 μ m thick sections. The sections were fixed with acetone on glass slides at -20°C for 8 min. They were treated with 0.3 % H₂O₂ in Tris-buffered saline (TBS) for 20 minutes to block endogenous peroxidase activity and treated with goat normal serum in TBS (1:66) for 60-90 minutes to reduce non-specific staining. The used primary antibodies and dilutions are listed in table 3. The primary antibodies were diluted with TBS 1 % Bovine albumin and incubated for 30- 45 minutes, except for AR which was incubated over night at 4°C. The primary antibodies were detected with the Vectastain ABC kit (Vectorlabs, Burlingame, CA) according to the manufacturer's protocol. The AR-primary antibody was detected with the EnVision/HRP ready-to-use detection reagent (Dako A/S, Glostrup, Denmark) according to the manufacturer's protocol. The tissue sections were further stained with diaminobenzidine solution and counterstained with Mayer's hematoxylin.

An IHC score was calculated from the α 2- and α 11-stained prostate cancer sections. The IHC score used in paper I is a combination of the staining intensity and its coverage area in the tissue (Luo et al. 2002). The staining intensity ranges from values 0 to 3. Intensity value 0 is negative, 1 is weak staining, 2 is moderate staining and 3 is strong staining. The coverage is defined as percentage (0-100 %) and is estimated for each intensity value (0-3). To calculate the IHC score, the percentages are multiplied with each intensity value. For example, 0 (intensity value) x 10

(%), 1 x 10, 2 x 20 , 3 x 60) = 230. The maximum IHC score is 300 (intensity 3 x 100 (%). The staining intensity was estimated semi-quantitatively and manually.

In paper IV, the immunofluorescence detection of CT16 was performed with the metastatic melanoma cell line SK-Mel-2, the stably CT16 transfected WM-266-4 cell line and its negative control (empty vector). A cell density of 20 000 cells/cm² on a glass coverslip was used. The cells were incubated overnight and labeled with the CT16-specific antibody (15 µg/ml) by an indirect immunolabeling method (Owen et al. 2010). The CT16 antibody was detected with Alex Fluor 488 goat anti-rabbit IgG (1:100, Invitrogen).

4.9 Time-resolved Fluoroimmunoassay (III)

In paper III the concentration of CT16 was determined in the sera of melanoma stage IV patients, in cell lysates or cell culture medium derived from several cancer cell lines. The measurement was based on a streptavidin-biotin system and europium fluorometric assay.

200 ng of a biotinylated CT16-specific antibody, suspended in DELFIA assay buffer (Perkin Elmer), was added to a 96-well streptavidin-coated microplate (Kaivogen, Turku, Finland). After an incubation of 60 minutes and washing with a 5 mM Tris-Cl buffer, the serum samples were applied to the plate. The samples were diluted in the DELFIA assay buffer, (1:1.5, total volume 100 µl), and added to the plate, which was incubated for 1 hour and washed twice with Tris-Cl buffer. 100 ng of a Europium-labeled CT16-specific antibody was added and incubated for 1 hour. After several washes, 200 µl of DELFIA Enhancement solution (Perkin Elmer) was applied to each well to develop the europium chelate fluorescence. After 45 min incubation, the fluorescence was detected with a Victor3 plate reader using excitation/emission wavelengths of 340/615nm. All incubations were performed at room temperature.

The measurement of S100B and LDH in serum was performed with a S100B ELISA kit (BioVendor, Heidelberg, Germany) and a Lactate Dehydrogenase assay kit (BioVision, Mountain View, CA), respectively, according to the manufacturer's instructions.

4.10 Transcriptome analysis (IV)

A transcriptome analysis (genome-wide gene expression microarray) was performed with CT16 knock down melanoma cells, CT16 overexpressed melanoma cells and their respective negative control cells. This was done to find possible alterations in gene expression caused by the inhibition and over-expression of CT16 in melanoma cells.

Total RNA was extracted from the following samples: CT16-siRNA1 transfected A2058 cells at 48 hour time point and WM-266-4 cells stably transfected with CT16 expression construct as well as their respective negative control cells. The negative control samples consisted of A2058 cells transfected with negative control-siRNA, and WM-266-4 cells transfected stably with an empty vector. Triplet cell culture plates were used for each cell sample. RNA was extracted from every cell plate and was analyzed with the gene expression array at the Finnish Microarray and

Sequencing Centre (FMSC), Turku Finland. At FMSC, the RNA was amplified, biotinylated and hybridized to Illumina's Sentrix Human-6 expression Bead chip and scanned with Illumina Bead array Reader, Bead scan software v 3.5.49 (Illumina Inc., San Diego, CA). The microarray data was normalized using the quantile normalization method in Bioconductor (www.bioconductor.org). The genes whose expression was altered were identified using the rank product algorithm (Bioconductor RankProd package).

4.11 Cell proliferation assays and real time monitoring of cells (IV)

Cell proliferation assays were performed with WM-266-4 cells transfected with stable CT16, and A2058 cells transfected with CT16-siRNA 1 and -siRNA 2, and their negative control cells in paper IV.

WM-266-4 cells transfected with CT16 (2500 cell/ well) and A2058 cells transfected with CT16 siRNA (10 000 cells/well) were seeded to a 96-well plate and cultured over night at 37°C. Six parallel samples were prepared. The cell proliferation assay was performed using a CellTiter 96 nonradioactive cell proliferation assay kit (Promega) according to the manufacturer's instructions. The produced solubilized formazan dye was measured at 570 nm with a 96-well plate reader. The proliferation of WM-266-4 cells was followed for 96 hours with 24 hour intervals, while the proliferation of A2058 cells was followed for 10 hours with 2.5 hour intervals.

The proliferation of the melanoma cells was additionally monitored in real time in paper IV. Real time monitoring was carried out by measuring the electrical impedance by xCELLigence (Roche and ACEA Biosciences Inc.). The electrical impedance is proportional to the cell number attached to the micro electrodes at the bottom of the well in an xCELLigence plate (96 E-plate™, Roche and ACEA Biosciences Inc.). The micro electrodes cover 80 % of the bottom of the well. To measure the impedance A2058 cells, transfected with CT16 siRNA1 and 2 (48 h), were seeded to the 96-well xCELLigence plate (10 000 cells/ well). The electrical impedance of the wells was measured every 10 minutes for 10 hours, followed by the addition of cisplatin (10, 50 and 100 µM in 100 µl DMEM) (Sigma-Aldrich, St. Louis, MO) to induce apoptosis. After the addition of cisplatin, the impedance measurements were continued for 30 hours. The impedance was measured and displayed as Cell Index (CI) values. Cisplatin can also be used for melanoma treatment (Daponte et al. 2005).

4.12 p53 conformation and stability analyses (IV)

Conformation and stability analyses of p53 were done to investigate if CT16 affects p53. Lysates from cultured WM-266-4 cells were incubated at 0, 30, 60 or 120 min at 37°C with recombinant CT16 or glutathione S-transferase (GST). The wild-type conformation-specific (active) p53 and the mutant conformation-specific (inactive) p53 were immunoprecipitated with the antibodies PAb1620 (Abcam) and PAb240 (Invitrogen), respectively. Total p53 was detected with Western blotting (p53 antibody, sc-126, 1:500, Santa Cruz Biotechnology).

The stability of p53 was analyzed by inhibiting protein synthesis with cyclohexamide (CHX, Sigma-Aldrich). 30 µg/ml of CHX was added to CT16- siRNA A2058 cells (48 h) during culturing and the cells were lysed after 0, 3 and 6 hours after the addition of CHX. The lysates were immunoblotted for total p53 (sc-126, 1:500, Santa Cruz Biotechnology).

4.13 Histone-H3-acetylation and DNA-methylation specific immunoprecipitation analyses (IV)

WM-266-4 cells stably transfected with CT16 and empty vector were used in the acetyl-histone H3- and methylated DNA specific immunoprecipitation analyses to investigate if there is connection between histone-H3 acetylation/DNA methylation and the genes regulated by CT16. The analyses were done using the Upstate Acetyl-Histone H3 Immunoprecipitation Assay Kit (Millipore, Billerica, MA) and the MethylAmp Methylated DNA Capture kit (Diagenode, Liege, Belgium) according to the manufacturers' protocols. The acetylation kit was used to immunoprecipitate transcriptionally active chromatin/gene promoters from the melanoma cells using antibody anti-acetyl-Histone H3. In the methylation kit, a monoclonal antibody targeting 5-methylcytosine was used to immunoprecipitate methylated genomic DNA from the melanoma cells. The immunoprecipitated samples were amplified with the WGA2 kit (Sigma), and purified with the GenElute PCR Cleanup kit (Sigma). The samples were analyzed at NimbleGen services using the human HG18 Deluxe Promoter HX1 chip array to detect active promoters. The array covered 30,000 gene promoters (Roche).

4.14 Preparation and analysis of nuclear and cytosolic fractions (IV)

Melanoma SK-Mel-2 cells were suspended in homogenization buffer together with a complete mini protease inhibitor tablet (Roche). The cells were homogenized with a Dounce homogenizer (Sigma-Aldrich) and centrifuged at 750 x g for 10 minutes. The supernatant was further centrifuged at 20,000 x g for 1 hour. After centrifugation, the supernatant contained the cytosolic proteins and the pellet contained the nuclei. The pellet was purified with two buffers. The pellet was suspended first in Buffer A: 10 mM HEPES, 10 mM KCl, 0.25 M sucrose, 10 mM MgCl₂, pH 7.9 and then in an equal volume of buffer B: 10 mM HEPES, 10 mM KCl, 0.35 M sucrose, 0.5 mM MgCl₂, pH 7.9, and then centrifuged at 1400 x g for 5 minutes. The purification step was repeated once. After purification, the pellet was mixed with a mini protease inhibitor tablet and homogenization buffer. The nuclear proteins were extracted by sonication and centrifugation at 18 000 x g for 10 min. Equal amounts of nuclear or cytosolic proteins were analysed by SDS-PAGE and Western blotting to detect CT16 and the cytosolic control protein MEK1/2 (Antibody, 9122, Cell Signaling Technology). All centrifugations were done at 4°C.

4.15 Statistical and Data analysis (I-IV)

The Tukey-Kramer test was used to calculate the statistical significance in α 2-expression and between CT16 and DKK-1 levels in the prostate cancer and melanoma skin metastasis samples,

respectively. Tukey-Kramer test was used since the amount of samples in each group was unequal. Pearson correlation was determined between the mRNA expression of collagen binding integrins and AR. Pearson correlation method is a parametric test and measures the strength of the linear relationship between normally distributed variables. Tukey-Kramer and Pearson correlation analyses were carried out using the SAS system software (SAS 8.2/9.2, SAS Institute Inc, Cary, NC). Wilcoxon-Mann-Whitney exact test was used to calculate the statistical significance of the differences in CT16 expression between melanoma and prostate cancer as well as in serum samples of melanoma and control patients. Wilcoxon-Mann-Whitney test is a non-parametric test, used when the assumption of normality is not required. Spearman correlation was determined between CT16 and S100B levels and between CT16 and LDH levels in melanoma serum samples. Spearman correlation is preferred when some values are not within normal distribution (outliers). Wilcoxon-Mann-Whitney and Spearman correlation analysis was run by using SPSS software, version 16.0 (SPSS, Chicago, IL).

The program Go Term Mapper (<http://go.princeton.edu/cgi-bin/GOTermMapper>) is a fast tool to describe the Gene Ontology of different genes. The program was used to describe the biology of our hit genes (differentially expressed genes) of the transcriptome analysis. The program categorized the genes into different biological processes (GO slims) and cellular components. DAVID Functional Annotation Tool was used to specifically identify the biological processes (<https://david.ncifcrf.gov/>).

5. RESULTS

5.1 Expression of collagen binding integrins in cancer cell lines, prostate cancer and advanced melanoma (I,II)

Only the α -subunits ($\alpha 1$, $\alpha 2$, $\alpha 10$ and $\alpha 11$) of the collagen binding integrins were analyzed in this thesis to specifically measure the expression of the $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$ and $\alpha 11\beta 1$. The α -subunits of the collagen binding integrins only form a heterodimer with one type of β -subunit, namely the $\beta 1$ subunit.

5.1.1 mRNA expression of collagen binding integrins in cancer cell lines

The mRNA expression of $\alpha 1$, $\alpha 2$, $\alpha 10$ and $\alpha 11$ was measured with qRT-PCR in 24 human cancer cell lines. β -actin was used as the reference gene. Integrin $\alpha 2\beta 1$ was the most frequent collagen binding integrin, and was the main receptor in 83 % (20/24) of the cell lines (Table 4). All metastatic prostate cancer cell lines (PC-3, LnCap and Du-145) and 2/3 melanoma cell lines (WM-115 and A2058) expressed $\alpha 2$ as their main collagen receptor. Saos (osteosarcoma) was the only $\alpha 2$ -negative cell line.

Table 4. Mean mRNA expression of $\alpha 1$, $\alpha 2$, $\alpha 10$ and $\alpha 11$ in 24 cancer cell lines, divided into three groups: melanoma, sarcoma and carcinoma.

Cancer Type	Cell line	Origin	mRNA (% of β -actin)			
			$\alpha 1$	$\alpha 2$	$\alpha 10$	$\alpha 11$
Melanoma <i>n=3</i>	WM-115	Skin melanoma, primary	0	0.6	0.2	0
	A2058	Melanoma, lymph node metastasis	3.0	5.2	0.2	0
	SK-MEL-5	Melanoma, lymph node metastasis	5.7	1.5	0.1	1.9
Sarcoma <i>n=5</i>	MG-63	Osteosarcoma, bone	1.2	28.3	0	3.2
	KHOS-240	Osteosarcoma, bone	1.4	1.9	0.2	1.5
	HT-1080	Fibrosarcoma, connective tissue	1.5	41.6	4.3	1.5
	SK-LMS-1	Leiomyosarcoma, vulva	0.5	8.6	0.1	0.6
	SAOS	Osteosarcoma, bone	6.5	0	0.3	9.5
Carcinoma <i>n=16</i>	UT-SCC-10	Squamocellular ca. head and neck region	0.6	25.5	0	0
	UT-SCC-15	Squamocellular ca. head and neck region	0.6	9.9	0	0
	NCI-H2170	Squamocellular ca. lung	0	3.2	0	0
	NCI-H520	Squamocellular ca. lung	0	1.2	0	0
	NCI-H1563	Non-small cell adenocarcinoma, lung	0.7	1.4	0	1.0
	NCI-H2228	Non-small cell adenocarcinoma, lung	1.8	34.1	0	0.2
	HT-29	Adenocarcinoma, colon	1.9	3.1	0	0
	SW-620	Adenocarcinoma, colon	0.2	1.7	0	0
	HeLa	Adenocarcinoma, uterine cervix	7.4	3.0	0	0
	PC-3	Prostate adenocarcinoma, bone met.	7.7	18.4	0.6	0
	DU-145	Prostate adenocarcinoma, brain met.	1.2	17.0	0.1	0.1
	LNcap	Prostate adenocarcinoma, lymph n met.	0.8	1.4	0.01	0.02
	MDA-MB-231	Breast carcinoma, pleural metastasis	3.5	25.8	0.8	0
	T47D	Breast carcinoma, pleural metastasis	0	4.4	0.4	0
	Tera-1	embryonal carcinoma	0.8	1.7	0	0.2
	Tera-1	embryonal carcinoma	3.4	2.2	0	3.0

The absence of $\alpha 2$ expression in Saos cell line is in agreement with our earlier data (Ivaska et al. 1999; Vihinen et al. 1996). Integrin $\alpha 1$ was the second most frequent integrin. If 1 % mRNA is considered a significant level of abundance, $\alpha 1$ was present in 2/3 melanoma, in 4/5 sarcoma

and in 7/16 carcinoma cell lines. All three metastatic prostate cancer cell lines expressed $\alpha 1$ as their second most abundant collagen receptor.

Integrin $\alpha 10$ was significantly expressed in only one cell line, namely fibrosarcoma HT-1080, which is derived from the fibrous connective tissue of the bone. In non-cancerous cells, $\alpha 10$ is mainly expressed in chondrocytes of cartilage, but has been also found in osteoblasts in bone (Camper et al. 2001; Bengtsson et al. 2001). Additionally, HT-1080 was the only cell line with significant amounts ($\geq 1\%$) of all four collagen binding integrins. None of the prostate and melanoma cell lines expressed significant amounts of $\alpha 10$. Integrin $\alpha 11$ was significantly expressed ($\geq 1\%$) in 1/3 melanomas, in 4/5 sarcomas and in 2/16 carcinoma cell lines. $\alpha 11$ was only significantly expressed ($\geq 1\%$) in one out of four lung adenocarcinoma cell lines. Integrin $\alpha 11$ is commonly overexpressed in tissues of lung adenocarcinoma compared to healthy tissues (Wang et al. 2002). The osteosarcoma Saos cell line, which was the only $\alpha 2$ -negative cell line, had the highest $\alpha 11$ expression of all cell lines. The $\alpha 11$ -expression in Saos was strikingly high (9.5 % of β -actin).

The presence of all four collagen binding integrins was much more widespread in sarcoma cell lines compared to carcinomas. The wider expression of collagen binding integrins in sarcoma depends on the higher frequency of $\alpha 10$ and $\alpha 11$ in sarcoma than in carcinoma. Four out of five (80 %) sarcoma cell lines (osteosarcoma, fibrosarcoma, leiomyosarcoma) were positive for $\alpha 10$, and all five sarcoma lines had detectable levels of $\alpha 11$. Only 31 % (5/16) and 38% (6/16) of carcinoma cell lines were positive for $\alpha 10$ and $\alpha 11$, respectively.

5.1.2 Levels of integrin $\alpha 2\beta 1$ decrease in parallel with progression of prostate cancer

The mRNA expression of $\alpha 2$ in 104 prostatectomies, including benign lesions and samples graded with Gleason scores 1-5, was screened with qRT-PCR. The qRT-PCR result showed that $\alpha 2$ mRNA levels decreased along with dedifferentiation in prostate cancer. The mean mRNA expression levels of $\alpha 2$ (% of β -actin) were 21.8 %, 15.6 % and 12.0 % in benign, Gleason 1-3 and Gleason 4-5 samples, respectively (I, Figure 3B). The difference in mRNA expression between benign lesions and Gleason grade 4-5 was significant ($p=0.0331$, Tukey-Kramer).

In accordance with the $\alpha 2$ mRNA expression levels, the protein staining of $\alpha 2$ diminished gradually and in parallel with poorer differentiation of the prostate carcinoma. The IHC scores decreased significantly from benign lesions to Gleason 5 lesions ($p=0.0001$, Kruskal-Wallis). The mean IHC scores were 228.8 in benign and 222.2 in low PIN tissues, while the scores were 194.5, 187.1, 132.7, 86.1 and 35.4 in Gleason grade 1-5 samples, respectively (I, Figure 3A). Integrin $\alpha 2$ was localized mostly in the luminal epithelial cells (table 5). The strongest intensity was found in the lateral plasma membranes of the luminal epithelial cells. Some occasional and irregular staining was seen in the basal cells.

Table 5. The expression and the location of integrins during prostate cancer

Integrin	Increased (↑) or decreased (↓) expression	Location in the prostate
$\alpha 2$	↓ at both mRNA ($p=0.0331$) and protein level ($p=0.0001$)	Luminal epithelial cells
$\alpha 11$	↑ at protein level ($p=0.029$, benign vs. carcinomas 1-5)	Luminal epithelial cells Stromal cells *

*was not evaluated

5.1.3 The expression of integrin $\alpha 11\beta 1$ is increased in prostate cancer

An IHC staining showed that $\alpha 11$ was localized in both epithelial cells and in stromal cells (table 5). However, the stromal intensity of $\alpha 11$ was very irregular and difficult to study, which led to evaluations only from the epithelial cells and not from the stromal elements. Some irregular occasional staining was also found in the basal cells. Integrin $\alpha 11$ was concentrated to the lateral and apical cell membrane in the luminal epithelial cells. No significant differences were detected between IHC scores and Gleason grades, but the highest levels of $\alpha 11$ were found in the poorest differentiated prostate carcinoma (Gleason 5). Additionally, the mean IHC score was significantly enhanced in the carcinoma group compared to benign lesions. The mean IHC scores in carcinoma and in benign samples were 148.3 and 115.3, respectively ($p=0.029$, Mann-Whitney U -test) (I, Figure 4B).

The mRNA expression of $\alpha 11$ was slightly enhanced in Gleason 4-5 samples compared to Gleason 1-3 carcinomas. This is comparable with the highest protein levels in the poorest differentiated samples. There was no significant difference or correlation between $\alpha 11$ and the histological groups. One logical reason for the non-significant result is the extensive variations in $\alpha 11$ expression in the stromal parts of the samples. The mean $\alpha 11$ mRNA expression levels (% of β -actin) were 2.4 %, 1.7 % and 2.2 % in benign, in Gleason 1-3 and in Gleason 4-5 lesions, respectively (I, Figure 4C).

5.1.4 $\alpha 1$ and $\alpha 10$ mRNA expression are increased in individual prostate cancer cases

In agreement with prostate cancer cell lines, $\alpha 1$ was the second most abundant collagen receptor in prostate carcinoma tissues. The mean mRNA expression levels of $\alpha 1$ were 25 %, 13.2 % and 15.9 % in benign samples, Gleason 1-3 and Gleason 4-5 carcinomas, respectively (I, Figure 6A). Integrin $\alpha 1$ levels did not correlate significantly with the Gleason grades. However, Gleason 5 lesions expressed the lowest $\alpha 1$ levels. The level of $\alpha 1$ was strikingly high in several patients.

The mean mRNA expression levels of $\alpha 10$ were 1.8 %, 0.9 % and 4.2 % (% of β -actin) in benign samples, Gleason 1-3 and in Gleason 4-5 carcinomas, respectively (I, Figure 6B). No significant correlations were found between the graded groups. The highest expression levels of $\alpha 10$ were detected in Gleason 4-5 carcinomas, but can be explained by the two exceptionally high $\alpha 10$ values seen in two patients. The high values of $\alpha 10$ were 124.8 % and 25.9 %. The patient with

an exceptionally high $\alpha 1$ mRNA level was not the same individual as the one with the high $\alpha 10$ value

5.1.5 Androgen receptor correlates with integrin $\alpha 2\beta 1$

The androgen receptor (AR) regulates the differentiation and proliferation of the cells in the prostate. It also stimulates the progression of prostate cancer cells. Prostate cancer is therefore usually treated by androgen deprivation therapy (Tan et al. 2015).

The mRNA expression levels of AR and the IHC index of AR in the nuclei were analysed in the benign and the prostate carcinoma samples. The AR nuclei staining could be determined in 78 tissue slides. The qRT-PCR analysis did not show any significant change in the AR mRNA levels. The mean mRNA expression of AR was 38.1 %, 27.7 % and 31.5 % (of β -actin) for benign, Gleason 1-3 and Gleason 4-5 prostate carcinomas, respectively (I, Figure 5A). The mean protein levels (IHC index) of AR were 43.9, 40.7 and 31.7 for benign, Gleason 1-3 and Gleason 4-5 prostate carcinomas, respectively (I, Figure 5B). The index decrease between benign samples and Gleason 4-5 samples was significant ($p= 0.046$), but not between benign and Gleason 1-3 or Gleason 1-3 and Gleason 4-5.

Correlations between AR and the collagen binding integrins were analysed. AR showed a good significant correlation with $\alpha 2$. The AR mRNA correlated positively with the $\alpha 2$ mRNA, especially in the benign group ($p=0.0001$) and in Gleason 4-5 group ($p=0.0001$) (I, Figures 5 C, E). This data suggests that AR positively regulates the expression of $\alpha 2$. This *in vivo* data supports the previous data that AR correlates positively with $\alpha 2$ in prostate cancer cells (Nagakawa et al. 2004; Evangelou et al. 2002). However, since our result did not show significant reduction of AR mRNA or protein levels during malignant transformation (control vs. G1-3), despite the significant decrease of $\alpha 2$, the malignant transformation seems to regulate $\alpha 2$ expression AR-independently.

5.1.6 mRNA expression of $\alpha 1$ and $\alpha 2$ were significantly higher than of $\alpha 11$ in advanced melanoma

In paper II, the quantity of $\alpha 1$, $\alpha 2$ and $\alpha 11$ in advanced melanoma was studied with qRT-PCR in 26 human melanoma tissue samples (stage III or IV). The qRT-PCR result showed that $\alpha 1\beta 1$ and $\alpha 2\beta 1$ were the most common collagen receptors in the melanoma lesions. Both mean and median expressions were calculated from the qRT-PCR analyses. For all 26 samples the mean levels of $\alpha 1$, $\alpha 2$ and $\alpha 11$ were 159, 425 and 55 (% of β -actin), respectively. The median levels of $\alpha 1$, $\alpha 2$ and $\alpha 11$ were 80, 55 and 15 (% of β -actin), respectively. The lower expression of $\alpha 11$ was significantly lower than the expression of $\alpha 1$ (0.0006 Wilcoxon signed-rank test) and $\alpha 2$ ($p 0.0002$ Wilcoxon signed-rank test). The mRNA expression of $\alpha 1$, $\alpha 2$ and $\alpha 11$ also correlated significantly with each other: $\alpha 1$ mRNA vs. $\alpha 2$ mRNA ($R= 0.826$, $p<0.0001$), $\alpha 2$ vs. $\alpha 11$ ($r=0.731$, $p=0.0003$) and $\alpha 1$ vs. $\alpha 11$ ($r=0.696$, $p=0.0005$), Spearman's rank correlation test. An IHC staining of $\alpha 1$ and $\alpha 2$ was performed to confirm the expression of the collagen receptors at the protein level (Data not shown). No correlation analyses were performed with the IHC samples. The

staining of $\alpha 2$ was localized mostly in the tumor cells, while $\alpha 1$ was present in both tumor cells and blood vessels. No staining for $\alpha 11$ was performed.

5.1.7 Impact on survival in advanced melanoma patients with high or low $\alpha 1$ -, $\alpha 2$ - and $\alpha 11$ mRNA

In paper II, we wanted also to analyse potential correlations between the levels of collagen binding integrins ($\alpha 1$, $\alpha 2$ and $\alpha 11$) and survival times in melanoma. Two patient groups for each integrin ($\alpha 1$, $\alpha 2$ and $\alpha 11$), making six groups altogether, were studied for three different survival time parameters (Kaplan-Meier survival curves). The two patient groups were categorized as 1) patients with high levels of $\alpha 1$ mRNA (alternatively $\alpha 2$ or $\alpha 11$) and 2) patients with low $\alpha 1$ mRNA levels (alternatively $\alpha 2$ or $\alpha 11$). A representative cut-off value (threshold value) was determined to distinguish between high and low mRNA groups and was made for each integrin. The cut-off value was defined by assigning different values to the cut-off point and determining the corresponding P values, using the Mantel-Cox (log rank) test to analyse the Kaplan-Meier survival curves. The cut-off values were 250, 200 and 75 for $\alpha 1$, $\alpha 2$ and $\alpha 11$, respectively. The three studied survival time parameters were a) survival after the first melanoma metastasis, b) survival after the initiation of chemoimmunotherapy and c) overall survival (timepoint from the primary diagnosis). The correlations between the survival time and the integrin levels were calculated with the Spearman's rank correlations test, where $p \leq 0.05$ is significant. The survival time was defined in months.

Corrections: Unfortunately, some figure numbers mentioned in article II are not connected with the correct figures. The figure numbers mentioned in this thesis are accurate and linked with right figures in article II.

5.1.7.1 Integrin $\alpha 2\beta 1$ has great impact on overall survival in advanced melanoma

Integrin $\alpha 2\beta 1$ was the most prominent integrin with survival impact. The overall survival time was strikingly longer in patients with low $\alpha 2$ mRNA levels (≤ 200) ($n=20$) than in patients with high $\alpha 2$ mRNA levels ($n=6$). The median length of overall survival was 53 months in patients expressing low $\alpha 2$, and 35 months in patients expressing high $\alpha 2$ ($p=0.033$) (II, Figure 3 C). The survival time after the first metastasis was almost 50 % longer in patients with low $\alpha 2$ levels than in patients with high $\alpha 2$ levels, with a median time of 30 months vs. 16 months in the respective groups ($p=0.048$) (II, Figure 3 A). The median survival after the initiation of chemoimmunotherapy was 14 months in patients expressing low levels of $\alpha 2$, and 8 months in patients expressing high levels ($p=0.040$) (II, Figure 3 B). Integrin $\alpha 2\beta 1$ was the only collagen receptor to have a significant difference in the overall survival time in advanced melanoma.

5.1.7.2 Low levels of $\alpha 1\beta 1$ predict enhanced survival after beginning of treatment and first metastasis

A significant difference in survival time after the first metastasis was observed with $\alpha 1$ mRNA. The survival time after the first metastasis was 6 months for patients expressing high $\alpha 1$ levels

(≥ 250) ($n=4$) and 31 months for patients with low $\alpha 1$ levels ($n=22$) ($p=0.002$) (II, Figure 2A). Furthermore, a significant difference was seen in survival after initiation of therapy. Patients with high $\alpha 1$ levels had a median survival of 3 months compared with 12 months for patients with low levels ($p=0.004$) (II, Figure 2B). The results of the overall survival analysis were not significant. The median overall survival time was 41 months for low $\alpha 1$ expressing patient group compared with 35 months for high $\alpha 1$ expressing group (II, Figure 2C).

5.1.7.3 Low levels of $\alpha 1181$ predict enhanced survival after beginning of treatment and first metastasis

Similarly to $\alpha 1$, high $\alpha 11$ mRNA levels were also connected with a significantly shorter survival time after the first metastasis and after the initiation of chemoimmunotherapy. The limit for a high $\alpha 11$ mRNA level was ≥ 75 (% of β -actin). The median survival after the first metastasis for patients with high $\alpha 11$ ($n=6$) was 16 months compared to 30 months for patients with low $\alpha 11$ levels ($n= 20$, $p= 0.002$) (II, Figure 4A). The median survival time after the initiation of chemoimmunotherapy was 8 vs. 14 months in patients with high $\alpha 11$ mRNA vs. low $\alpha 11$ mRNA levels ($p= 0.009$) (II, Figure 4B). The overall survival analysis did not show any significant differences (II, Figure 4 C).

5.1.7.4 Lower levels of $\alpha 261$ and $\alpha 1181$ correlated with diffuse expression of Bcl-2

The studies in paper II also showed that low mRNA expression of $\alpha 2$ and $\alpha 11$ were related to a diffuse protein expression of Bcl-2 in the metastatic melanoma tissues (II, Figure 6). (The expression of Bcl-2 had previously been investigated with IHC in another study (Vlaykova et al. 2002). A diffuse Bcl-2 expression means in this case a positive Bcl-2 expression throughout the whole melanoma tumor. Bcl-2 is known to regulate apoptosis. Bcl-2 can inhibit cell death in cancer and contribute to resistance of chemotherapy, but it can also induce apoptosis (Jansen et al. 2000; Czabotar et al. 2014). A melanoma tissue microarray study has demonstrated that enhanced levels of Bcl-2 are linked to improved survival in metastatic melanoma (Divito et al. 2004). Our result supports the microarray study since positive staining of Bcl-2 was connected to lower levels $\alpha 2$ and $\alpha 11$, which promoted longer survival in melanoma patients in our survival analyses. Additionally, high levels of $\alpha 1$ and $\alpha 2$ were associated with negative staining of Bcl-2 in the melanoma tumors (II, Figure 6).

5.2 Expression of Cancer Testis Antigen 16 (CT16) in cancer cell lines, prostate cancer and advanced melanoma (III, IV)

CT16 has been classified as a prostate CT antigen due to its homology with other PAGE proteins (Prostate-associated genes). CT16 has therefore been included in the PAGE family. CT16 is also known as PAGE-5. However, there are no published data on the expression of CT16 in either prostate cancer or in normal prostate tissue. There has either been no detection of CT16 at protein level (CT database).

5.2.1 CT16 is not expressed in prostate cancer

In this thesis, CT16 was not detected in primary prostate cancer. CT16 was neither present at mRNA level or at the protein level. CT16 mRNA expression was analysed in 30 human prostate carcinomas, (graded from Gleason 2 to Gleason 5). The samples originated from the same collection of prostate cancer samples that were analysed in paper I. The qRT-PCR analysis revealed that none of the 30 prostate carcinomas had any significant mRNA expression of CT16 (III, Figure 1). CT16 mRNA or CT16 protein were neither present in the metastatic prostate cancer cell lines PC-3 or DU-145 (III, Figure 2C,D). No protein staining for CT16 was seen in primary prostate cancer tissue (IV, Figure 1C). Additionally, no hits for CT16 were registered in normal prostate when a CT16-screening was performed in two prostate-derived expressed sequence tags (EST) libraries. The cDNA libraries used were dbEST of (<http://www.ncbi.nlm.nih.gov/dbEST/>) (Boguski, Lowe, and Tolstoshev 1993) and the human prostate expression database (PEDB) (<http://www.pedb.org>) (Nelson et al. 2002). These results suggest that CT16 cannot be classified as a prostate-associated gene.

As other CT antigens, CT16 was detected in normal testis by IHC, and was served as a positive control (IV, Figure 1D). CT16 was enriched in the spermatogonia of the testis, which are undifferentiated cells that initiate spermatogenesis and undergo mitosis to become spermatocytes. The localization of CT16 in spermatogonia is in accordance with the expression sites of other CT-X antigens. In normal tissues, many CT-X antigens, such as MAGE and NY-ESO-1, are expressed in spermatogonia (Simpson et al. 2005).

5.2.2 CT16 is associated with melanoma

We wanted also to investigate CT16 in melanoma, since CT16 mRNA had been observed once in melanoma in a study by Scanlan et al. (Scanlan et al. 2002a). In this thesis, CT16 was studied in advanced melanoma tissues, several cancer cell lines and in serum samples of metastatic melanoma patients

5.2.2.1 CT16 is frequently expressed in tissues of advanced melanoma

The qRT-PCR analysis demonstrated that as much as 50 % (11 out of 22) of the metastatic melanoma lesions had significant detectable levels of CT16 mRNA (≥ 0.3 % of β -actin) (Figure 2A). Several tissue lesions with different amounts of CT16 mRNA (range between 0 % - 28 %) were IHC stained for CT16. The staining intensity of CT16 correlated significantly with the qRT-PCR data (Figure 2B, $r=0.966$, $p=0.002$). The staining intensity of CT16 was quantified from the pixel intensity of the grey scale IHC-pictures.

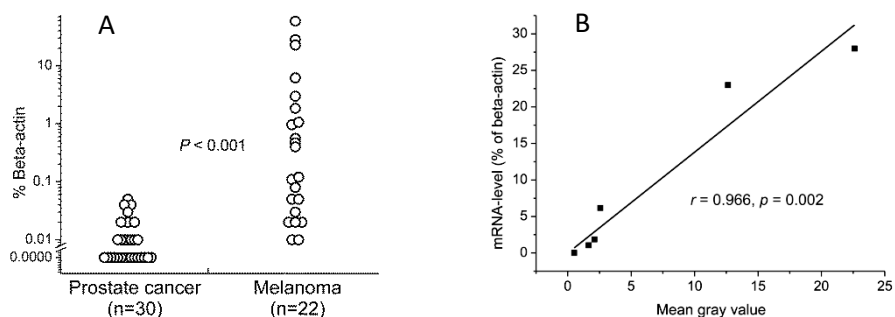


Figure 2 qRT-PCR analysis of CT16 mRNA (% of β -actin) and its correlation with CT16 protein levels. **A.** CT16 mRNA (% of β -actin) in human prostate cancer and in metastatic melanoma. **B.** mRNA levels of CT16 correlate with protein levels of CT16 in metastatic melanoma.

The qRT-PCR revealed also that CT16 was present in melanoma *in vitro*. Three out of five metastatic melanoma cell lines: A2058, SK-Mel-2 and SK-Mel-5 had significant levels of CT16 (III, Figure 2 D). The melanoma cell lines WM-115 and WM-266-4, which are derived from the same patient, did not express CT16 mRNA (III, Figure 2D). Immunoblotting of the same cell lines gave comparable results (III, Figure 2C). In addition to melanoma, CT16 was also enriched in one fibrosarcoma cell line HT-1080 (III, Figure 2 C,D).

Taken together, the results indicate that CT16 is a common melanoma CT antigen. CT16 expression has been reported once before in melanoma with qRT-PCR, but with a lower frequency (22 %, 4 out of 18 melanoma tissues) (Scanlan et al. 2002a) (CT database).

5.2.2.2 Cellular location of CT16

The cellular location of CT16 was presented for the first time in paper IV. CT16 was detected in both the cytoplasm and the nucleus of melanoma cells. However, the expression was stronger in cytoplasm than in nucleus. This conclusion is based on the detection of CT16 in the cytosolic and nuclear fractions derived from SK-Mel-2 cells (IV, Figure 2B) and on immunofluorescence staining in SK-Mel-2 and in the CT16-transfected melanoma cell line WM-266-4 (IV, Figure 2A). The results from the cultured cells also indicated that CT16 is secreted by cancer cells. CT16 was detected in the culture media of melanoma and fibrosarcoma cell lines by the sensitive time-resolved immunofluoroassay. The secreted concentration of CT16 was in accordance with the cellular level of CT16 (III, Figure 4A, B). The strongest cellular and secreted expression of CT16 was found in SK-Mel-2. However, it cannot be ruled out that the positive CT16 detection in the culture media is due to floating dying cancer cells.

5.2.3 CT16 is frequently expressed in sera of advanced melanoma

A time-resolved immunofluoroassay with an optimized Europium-labelled CT16 antibody was used in paper III to measure the CT16 concentration in the serum samples of 23 patients with stage IV melanoma. The melanomas were of cutaneous (n=15), uveal (n=3) or unknown primary

origin (n=3) (III, Table 3). The serum samples to measure CT16 levels were collected before the patients received treatment for the melanoma.

The time-resolved immunofluoroassay showed that CT16 was frequently expressed in the sera of advanced melanoma patients (III, Figure 3B). Detectable CT16 concentrations (> 0.3 ng/ml) were found in 61 % of the serum samples (14 out of 23), which are comparable with the presence of CT16 mRNA in 50 % of the melanoma tissues (chapter 5.2.2.1). CT16 levels did not significantly correlate with a specific type of distant metastasis. Interestingly, CT16 acted also as an independent biomarker when compared to the well-established and common biomarkers LDH and S100B. CT16 did not correlate with either LDH ($p=0.233$) or S100B ($p=0.677$) (III, Figure 3D, F). The concentrations of LDH were calculated as IU/l and S100B as pg/ml (III, Figure 3 C,E). The negative control sera (nine healthy donors), which was collected by University of Turku, did not express any CT16 (<0.3 ng/ml). The commercial control group, reported to have sera from 20 healthy donors, had one CT16-positive sample. The reason for the positivity is unknown, but could be explained by the presence of an undiagnosed cancer at the time of the donation of the sera.

5.2.3.1 CT16 in sera during melanoma treatment

CT16 levels were measured in follow-up sera samples of four metastatic melanoma patients (Patient A, B, C and D). The follow-up sera samples were obtained during their melanoma treatment. Patient A, B and C received the same first-line treatment consisting of dacarbazine, bevacizumab and interferon- α 2a while patient D received a second-line treatment consisting of carboplatin, vinorelbine, interleukin-2 and interferon- α 2b. This analysis was done to investigate a potential connection between CT16 concentrations and differences in disease progression. S100B was measured as a reference. In all four individuals, CT16 concentrations responded to alterations in the melanoma condition (III, Figure 5A-D). (The S100B concentrations stayed similar in three cases (patient B, C and D) even though disease progression occurred).

For example: Patient B responded well to the melanoma treatment and the CT16 level dropped and stayed low (0.26-0.42 ng/ml) for almost a year (Figure 3). When the CT16 concentration had risen a bit at week 57, disease progression had been observed. When CT16 increased remarkably at week 68, more lung metastases were observed in a CT scan. In contrast, S100B levels appeared low the entire follow-up time. The patient started second-line treatment and no further serum samples were obtained. In patient C, the outcome was similar (Figure 3): The patient responded well to treatment and the CT16 level dropped and stayed very low until week 28, when disease progression was detected in a CT-scan. The following week, the CT16 level was elevated. The concentration of S100B was low throughout the entire time of the treatment. Second-line treatment was started and no more serum samples were obtained.

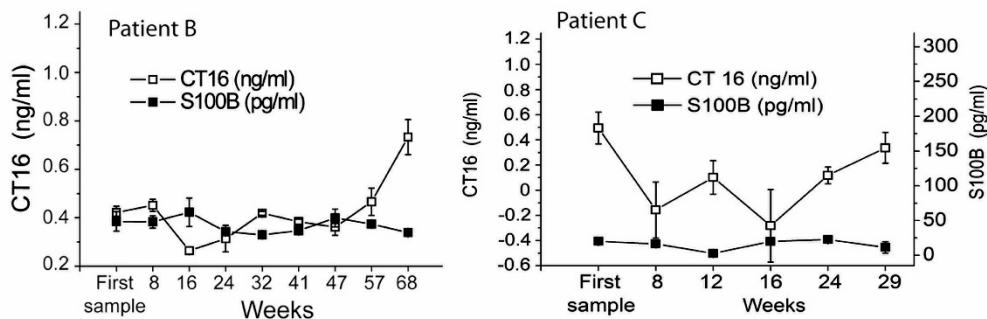


Figure 3. CT16 and S100B concentrations in follow-up sera samples of metastatic melanoma patients B and C during melanoma treatment

According to the results from the CT16 analyses in the sera and tissue analyses, CT16 can be considered as a promising melanoma biomarker. The frequency of CT16 is high in melanoma, as it can be detected in more than half of the sera samples and in 50 % of tissue samples. Additionally, CT16 is independent when it is compared to the commonly used LDH and S100B serum markers. Further, CT16 shows potential to be a valuable response marker for metastatic melanoma treatment. However, more follow-up studies during treatment are needed to draw conclusions.

5.2.4 The biological role of CT16

The biological role of CT16 is unknown. Two types of CT16-melanoma cells were used in the characterisation studies: 1) WM-266-4 cells stably transfected with CT16 expression vector and its negative control (WM-266-4 cells transfected with empty vector) 2) a CT16-siRNA transfected A2058 cells (CT16 knock down) and its negative control (A2058 cells transfected with negative siRNA). Before using CT16 siRNAs, the siRNAs were tested for their specificity. The protein levels of the common CT antigen MAGE-A1 and the CT16-related GAGE-1 were not affected by CT16 siRNAs (IV, Supplementary (S) 3D). PAGE2B, which shares the highest sequence identity with CT16, was not expressed in the melanoma cell lines: SK-Mel-2, WM-115, WM-266-4 and A2058. The PAGE2B expression test was done with RT-PCR. cDNA of human testis was used as a positive control for PAGE2B (IV, S 3E).

5.2.4.1 CT16 inhibits drug induced apoptosis

The results from the proliferation assay, based on formazan dye, showed that CT16 no impact on proliferation, neither in the stably CT16 transfected melanoma cells nor in the CT16 knock down cells (IV, Figure 3A,B). In contrast, CT16 had a significant effect on drug induced apoptosis. The apoptosis was followed up by monitoring the proliferation in real time with an impedance-based assay instrument (xCELLigence™). After addition of the apoptosis inducing drug cisplatin to the CT16 siRNA treated A2048 cells, cell death increased significantly compared to negative control cells. The most significant difference in cell growth was seen 14 hours after the addition

of 50 μM cisplatin (IV, Figure 3D). The role of CT16 as an anti-apoptotic protein was further verified with the detection of elevated levels of cleaved caspase-3 in CT16 knock down cells (IV, Figure 3C).

5.2.4.2 CT16 regulates genes associated with survival

Gene expression microarray was done to identify biological processes and CT16-associated genes, whose expression was altered in response to CT16 modulation in cultured melanoma cells. Two separate screens were done at the Finnish Microarray and Sequencing Centre in Turku. One screen was run with triplet RNA samples of CT16-transfected WM-266-4 melanoma cells and the negative control cells. The second screen was performed with triplet RNA samples of CT16 siRNA A2058 melanoma cells, and the negative control cells. By analysing two different melanoma cells lines with triplet samples strengthens the credibility of the gene list.

A representative and shorten gene list was created based on the two full length gene lists received from the two microarray screens. The representative gene list contained only the genes that were significantly altered by both stably CT16-transfected WM-266-4 cells and CT16 siRNA-transfected A2058 cells (false discovery rate (FDR) < 0.1). Eleven genes met the criteria and are listed in table 6.

Table 6. List of genes which are downregulated or upregulated by CT16 overexpressed WM-266-4 cells or by CT16 siRNA knock down A2058 cells. The up- and downregulation are described as fold change compared to negative control

Gene Symbol	Gene name	CT16 overexpression	CT16 knock down	Cancer relevance
DKK-1	Dickkopf homolog 1	-2.00	2.81	Apoptosis activator and inhibitor of invasiveness in melanoma (Kuphal et al. 2006, Chen et al. 2012)
CYB5R2	Cytochrome b5 reductase 2	2.81	-1.88	<i>Not known</i>
RAMP1	Receptor activity modifying protein 1	1.75	-1.7	Promotes proliferation and tumorigenesis in prostate ca. (Logan et al. 2013)
FABP7	Fatty acid binding protein 7, brain	1.54	-1.63	Involved in melanoma proliferation and invasion (Goto et al. 2006, Šlipicevic et al. 2008)
RBPM5	RNA binding protein with multiple splicing 2	2.86	-1.56	<i>Not known</i>
MT2A	Metallothionein 2A	2.8	-1.53	Protects against ROS-mediated cell death (Reinecke et al. 2006)
SERPINA5	Serpin peptidase inhibitor, member 5 (Synonyms: PAI-3, PCI)	4.04	-1.47	Increases motility in breast cancer (Palmieri et al. 2002). Inhibits tumor growth independent of protease-inhibitory activity, but is also able to promote metastasis by its protease-inhibitory activity in melanoma (Akita et al. 2015)
RRP7A	Ribosomal RNA processing 7 homolog A	1.78	-1.42	<i>Not known</i>
IL-8	Interleukin 8	3.71	-1.42	Promotes tumor growth and angiogenesis in melanoma (Luca et al. 1997, Ria et al. 2010) Inhibits chemotherapy induced apoptosis in prostate cancer (Wilson et al 2008)
CDC14B	CD14 cell division cycle 14 homolog B	1.89	-1.38	<i>Not known</i>
ARPC1B	Actin related protein 2/3 complex, 1B	1.73	-1.34	<i>Not known</i>

The results demonstrated that CT16 regulates genes associated with cell survival and cancer progression. According to previous studies, six genes on the list are connected to cancer progression. Three of those six genes are associated to apoptosis, which are Dickkopf-1 (DKK-1), metallothionein 2A (MT2A) and interleukin 8 (IL-8). DKK-1 was the only gene downregulated by CT16. DKK-1 is a known inhibitor of the canonical Wnt-pathway and is suppressed in melanoma cell lines and melanoma tissues (Kuphal et al. 2006). DKK-1 has been found to induce

apoptosis in melanoma *in vitro* (Mikheev et al. 2007). The second apoptosis related gene MT2A was upregulated by CT16, and it inhibits ROS (Reactive Oxygen Species)-mediated apoptosis (Reinecke et al. 2006). IL-8, which was also upregulated by CT16, is known as a melanoma progression gene and decreases apoptosis by transcriptional upregulation of c-FLIP in prostate cancer *in vitro* (Peng et al. 2007; Wilson et al. 2008; Luca et al. 1997; Ria et al. 2010). The genes FABP7, SERPINA 5 and RAMP1 are associated with tumor progression (Logan et al. 2013; Slipicevic et al. 2008; Palmieri et al. 2002; Akita et al. 2015) and were upregulated by CT16 overexpression.

A gene ontology-analysis (cellular components and biological processes) of the differentially expressed genes was performed with GoTermMapperTool (<http://go.princeton.edu/cgi-bin/GOTermMapper>). Genes, with a false discovery rate (FDR) of <0.05, collected from the full length gene list were used. The study was done separately for the genes affected by CT16 knock down and for the genes affected by CT16 overexpression. The result showed that the cell components were similar between the CT16 knock down experiment and the CT16 overexpression experiment (IV, Figure 4). Likewise, the biological processes were similar between the CT16 knock down experiment and the CT16 overexpression experiment (IV, Figure 4).

The biological processes associated with the differentially expressed genes were specifically identified. The processes were identified in a functional annotation study by using DAVID bioinformatics resource analysis. The full length gene list (FDR<0.05) received from the microarray screens was used to identify the processes. The results from the analysis demonstrated that the upregulated genes caused by CT16 overexpression were significantly enriched in survival process or in processes related to inflammation, wound healing, chemical homeostasis and response to stimulus (IV, Table 1). The genes whose expression got decreased by CT16 overexpression were significantly associated with antigen processing and peptide/antigen presentation via MHC class II (FDR<0.05). The genes, whose expression was altered by CT16 knock down, were not linked with any significant biological process within FDR < 0.05.

5.2.4.3 CT16 is a negative regulator of DKK-1 in melanoma

DKK-1, which is an inhibitor of the Wnt-signaling pathway, was further investigated due to its role in activating apoptosis in melanoma and its downregulation in melanoma and other cancer types (Kuphal et al. 2006; Mikheev et al. 2007). DKK-1 inhibits also the invasion activity of melanoma cells *in vitro* (Chen et al. 2012). The extracellular DKK-1 inhibits the canonical Wnt signaling through its interaction with cell receptor LRP6 (Low-density lipoprotein receptor-related protein 6) at the cell surface (Semenov et al. 2001).

In accordance with our result of the gene microarray screens, the upregulation of DKK-1 mRNA in melanoma cells treated with CT16 siRNAs was detected with qRT-PCR analysis. Likewise, the downregulation of DKK-1 mRNA in CT16 transfected melanoma cells was confirmed with qRT-PCR (IV, Figure 5A,B). Upregulation of extracellular DKK-1 at protein level was also observed from cultured medium of CT16 knock down cells (IV, Figure 5C).

The expression of DKK-1 mRNA was further screened in 20 advanced melanoma tissues samples that were divided into two groups: 1) High CT16 expressing melanomas (>22 %, n=3) and 2) low CT16 expressing melanomas (<7%, n=17). Interestingly, a significant correlation between CT16 and DKK-1 was found (IV, Figure 5 D) ($p < 0.05$). High CT16 expressing melanomas expressed low levels of DKK-1 mRNA (average = 0.07 % DKK-1 mRNA (of β -actin)) and low CT16 expressing melanomas had high levels of DKK-1 (average= 7.5 % DKK-1 mRNA (of β -actin)). These results indicate that CT16 acts as a negative regulator of DKK-1.

5.2.4.4 CT16 is independent of p53 and DNA methylation

Some CT-X antigens function in a p53 dependent way, such as MAGE, which is thought to suppress p53-dependent apoptosis (Yang et al. 2007). According to the results in paper IV, p53 is not involved in regulating the function of CT16. The mRNA level of p53 stayed at the same level in the SK-mel-2 cell line and in CT16 overexpressing WM-266-4 cell line compared to its negative control and wild type cells (IV, Supplementary Figure (S) 5B). No difference was seen in the stability of p53 when protein synthesis was inhibited with CHX in CT16 siRNA cells (IV, S 5E). The independency of p53 was further confirmed when no conformational change of p53 (inactive or active) was seen in the crude extract prepared from WM-266-4 cells (IV, S 5D). Additionally, the upregulation of DKK-1 mRNA was seen in Saos-2 cells after the downregulation of CT16. Saos-2 cells are known not to express p53 (IV, S 5C).

Some CT-X antigens are known to regulate genes (gene expression) through DNA methylation and histone H3 acetylation. Therefore, CT16 was investigated with a DNA methylation- and histone H3 acetylation kit, using CT16 overexpressed WM-266-4 melanoma cell line. Interestingly, the histone acetylation specific immunoprecipitation data in paper IV indicated a correlation between Histone H3 acetylation and the expression of the CT16 regulating genes. The suggestion is made based on differences in the number of acetylated genes in the CT16 overexpressing WM-266-4 cell line compared to its negative control (IV, table S1). However, it cannot be ruled out that this is due to histone H3 acetylation, which doesn't involve CT16. The immunoprecipitation data on DNA methylation showed no connection with genes regulated by CT16, which indicates that CT16 does not regulate the differentially expressed genes through DNA methylation (IV, S6).

6. DISCUSSION

6.1 Collagen binding integrins in prostate cancer

6.1.1 Collagen binding integrins were frequently expressed in sarcoma cell lines

This qRT-PCR analysis was the first broad expression study of $\alpha 10$ and $\alpha 11$ in cancer cell lines. All four subunits of collagen binding integrins ($\alpha 1$, $\alpha 2$, $\alpha 10$ and $\alpha 11$) were most frequently expressed in sarcoma cell lines, compared to carcinomas and melanomas. In addition to abundant levels of $\alpha 1$ and $\alpha 2$, most sarcoma cells expressed $\alpha 11$ and some $\alpha 10$, while many carcinomas showed no expression of $\alpha 10$ or $\alpha 11$. The expression of $\alpha 10$ and $\alpha 11$ in mainly sarcomas may partly be explained by their expression in normal chondrocytes in cartilage ($\alpha 10$), and in normal fibroblasts in bone ($\alpha 11$) (Camper et al. 2001; Popova et al. 2007). Melanoma cell lines did not either express high amounts of $\alpha 10$ and $\alpha 11$. However, one metastatic melanoma cell line (SK-Mel-5) had significant levels of $\alpha 11$, which are in accordance to the positive levels of $\alpha 11$ in metastatic melanoma tissues in paper II.

6.1.2 Integrin $\alpha 2\beta 1$ is the main collagen receptor in cancer cell lines

The integrin $\alpha 2\beta 1$ seems to be essential for the progression of cancer, since $\alpha 2\beta 1$ was clearly the main collagen receptor in the cancer cell lines. Of 24 cancer cell lines, 18 cell lines expressed $\alpha 2$ as their main α -subunit of the collagen receptors. However, the frequent expression of $\alpha 2$ may also depend on cell culture conditions, which may favor $\alpha 2$ -positive cells. Integrin $\alpha 2$, which is known to be downregulated in breast cancer *in vivo*, has been shown to promote cell proliferation in cultured breast cancer cells (Ramirez et al. 2011; Klekotka et al. 2001). In our qRT-PCR analysis, $\alpha 2$ was the main collagen receptor in both of the investigated metastatic breast cancer cell lines, MDA-MB-231 and T47D. Integrin $\alpha 2$ was additionally highly expressed in the metastatic prostate cell lines derived from bone and brain, but not from lymph nodes. A microarray study has shown that integrin $\alpha 2$ promotes specifically metastases to bone compared to lymph nodes (Sottnik et al. 2013). Integrin $\alpha 2$ enhances bone metastases by binding to especially collagen type I (Hall et al. 2006).

Integrin $\alpha 1$ was expressed in 4/5 of the sarcomas and in 7/16 of the carcinomas (when 1 % of β -actin mRNA level is considered as significant expression). The levels of $\alpha 1$ varied a lot in our carcinoma cell lines and showed no clear correlation with any specific carcinoma type. In normal tissues, integrin $\alpha 1$ is expressed predominantly in mesenchymal cells, which are the origin of sarcomas. It is suggested that sarcomas are derived from mesenchymal stem cells (Tolar et al. 2007; Xiao et al. 2013). An interesting observation was done in the osteosarcoma cell line Saos. The Saos cells, which are known not to express $\alpha 2$, had the highest level of $\alpha 11$ mRNA (9.5 % of β -actin) of all cell lines. This result is comparable to our data in the prostate cancer tissues, where $\alpha 2$ -reduction was combined with an increase in $\alpha 11$ levels. A significant expression of $\alpha 11$ was also discovered in the fibrosarcoma cell line HT1080. After that, Lu and colleagues have demonstrated that TGF- $\beta 1$ enhances the transcription of $\alpha 11$ in HT1080 cell line. They identified also the TGF- $\beta 1$ responsive promoter sites of $\alpha 11$ (Lu et al. 2010). Integrin $\alpha 11\beta 1$ is the main collagen receptor in fibroblasts during embryonic development (Popova et al 2007).

6.1.3 The expression levels of $\alpha 2\beta 1$ and $\alpha 11\beta 1$ are altered during the progression of prostate cancer

The mRNA levels of $\alpha 2$ decreased gradually during the progression of prostate cancer. The decrease was further confirmed, when a significant reduction in $\alpha 2$ protein level was shown. The $\alpha 2$ -expression was localized mainly to the epithelial cells. This downregulation of $\alpha 2$ expression is in accordance with other prostate cancer studies. Bonkhoff and colleagues observed downregulation of $\alpha 2$ in WHO grade I and grade II prostate tumors (Bonkhoff, Stein, and Remberger 1993). In 2011 a microarray database study reported a decline of $\alpha 2$ mRNA levels in primary tumors and metastases compared to healthy prostate controls (Ramirez et al. 2011). The downregulation of $\alpha 2$ during the progression of prostate cancer is similar to the expression pattern in breast cancer, where $\alpha 2$ levels are remarkably decreased (Zutter et al. 1995). A re-expression of $\alpha 2$ in breast cancer cell lines led to enhanced epithelial phenotype with more cell adhesions and less capacity of invasion and motility *in vitro*. Re-expression of $\alpha 2$ resulted also in larger tumor volume in mouse xenografts (Zutter et al. 1995). The significant downregulation of $\alpha 2$ in the epithelial cells of the malignant prostate gland strongly indicates that $\alpha 2$ is also important for the maintenance of the glandular prostate structure. The genetic mechanism behind the dysregulation of $\alpha 2$ during cancer progression is unknown. For example, gene deletions of $\alpha 2$ have not been reported in cancer tissues even though $\alpha 2$ is located on chromosome 5q23, which is a common site of other gene deletions in some cancer types such as breast cancer and renal cell carcinoma (Korenaga et al. 2005; Bergamaschi et al. 2006)

Even though $\alpha 2$ is downregulated in the locally advanced prostatic adenocarcinoma, this does not exclude the possibility that $\alpha 2$ is an important promoter of the malignant process in advanced prostate cancer. As previously mentioned, integrin $\alpha 2$ has been characterized as a putative prostate cancer stem cell marker together with CD44⁺ and CD133⁺. The three markers are present in a small subpopulation of the prostate cancer cells (0.1 %) (Collins et al. 2005). Cancer stem cells are capable for unlimited self-renewal and are thought to be the initiators of tumor relapse, metastasis and resistance to chemotherapy (Gupta, Chaffer, and Weinberg 2009). Integrin $\alpha 2$, CD44⁺ and CD133⁺ are enhanced in clinical CRPC (Pfeiffer et al. 2011). A protein expression of > 5 % of prostate cancer stem cell markers $\alpha 2$ + c-Met or $\alpha 6$ + c-Met in primary prostate cancer predicts the development of bone metastases (Colombel et al. 2012).

Integrin $\alpha 11$ was clearly expressed at the mRNA level, and the IHC analysis confirmed the presence of $\alpha 11$ protein in the prostate cancer samples. The epithelial staining of $\alpha 11$ was quantified, and the most intensive staining was seen in the lesions of the highest grade. Integrin $\alpha 11$ had not been investigated in prostate cancer before and this result indicates that $\alpha 11$ localized in the epithelial cells may partially replace $\alpha 2$. The switch may have an important influence on the progression of the prostate cancer. The collagen binding to $\alpha 11$ instead of $\alpha 2$ may lead to changes in the signaling events. However, putative differences between $\alpha 2$ and $\alpha 11$ are very poorly known. A shift from $\alpha 2$ to $\alpha 11$ may reflect EMT phenomenon during prostate cancer progression. Integrin $\alpha 2$ is usually localized in epithelial cells, while integrin $\alpha 11$ is generally known to be expressed in mesenchymal and stromal cells (Tiger et al. 2001; Popova et al. 2007). Integrin $\alpha 2$ and $\alpha 11$ regulate the survival of normal mesenchymal stem cells through collagen type I (Popov et al. 2011). $\alpha 11$ is important for EMT in mammary gland epithelial cells by being a receptor for the ECM protein tenascin-X through the FBG domain and eliciting

latent TGF- β (Alcaraz et al. 2014). In esophageal squamous cell carcinoma α 11 directly interacts with CCN-1 (Cysteine-rich angiogenic inducer 61), which promotes activation and nuclear translocation of beta-catenin (Chai et al. 2012). CCN-1 is a critical activator of cell proliferation in prostate cancer *in vitro* and nuclear translocation of beta-catenin is a common process in cancer (Franzen et al. 2009).

Integrin α 11 was also localized clearly in the stroma by IHC, even though the expression levels varied greatly. However, the expression of α 11 in stroma may explain the reason why there was no great difference in the mRNA levels of α 11 in the Gleason groups. After our detection of α 11 in stroma, α 11 has been detected in stromal fibroblast cells in NSCLC (Zhu et al. 2007). It has been shown that integrin α 11 is overexpressed in cancer-associated fibroblasts and it stimulates the growth of the lung tumors *in vivo* through tumor-stroma interactions by co-operation with IGF-2 (Zhu et al. 2007). In lung cancer, stromal α 11 also increases collagen reorganization and stromal stiffness (Navab et al. 2015). Integrin α 11^{+/-} mice are also more susceptible to metastasis in brain, lungs, kidney and bone when compared to α 11^{+/-} or α 11^{-/-} mice (Navab et al. 2015). Integrin α 11 is also upregulated by TGF- β 1 in the myofibroblasts (cancer-associated fibroblasts) of the stroma (Honda, Yoshida, and Munakata 2010). Integrin α 11 is functioning as a critical activator of myofibroblast differentiation since it stimulates the expression of α -smooth muscle actin (α -SMA) which is a marker for the myofibroblasts (Carracedo et al. 2010). Myofibroblast differentiation stimulates cancer progression by reorganization of the collagen matrix and by enhancement of the tumor-stroma interactions and the tissue invasion. In mouse fibroblasts, collagenase MMP-13 and cathepsin K are active participators in the α 11-dependent reorganization of the collagen matrix (Barczyk et al. 2013).

Based on the latest α 11-studies in stromal fibroblasts, and on our α 11-detection in stroma and epithelia of prostate cancer, α 11 seems to be an important tumor-stroma interactor in the progression of human prostate cancer. It has been reported that a reactive stroma with enhanced expression of α -SMA and vimentin is present in prostate cancer (Tuxhorn et al. 2002). Integrin α 11 was, however, absent from our metastatic prostate cancer cell lines, PC-3, DU-145 and LnCap as well as in three out of our four lung cancer cell lines. This absence may be explained by the cell culture conditions, which may favor α 2-expression. It can for example be due to stromal factors, e.g. lack of growth factors or cytokines.

6.1.4 AR - a positive regulator of α 2 β 1

Since two *in vitro* studies in prostate cancer have reported co-expression between α 2 and AR (Nagakawa et al. 2004; Evangelou et al. 2002), we wanted to investigate if the possible connection could also be observed in prostate cancer tissues. Interestingly, the qRT-PCR results revealed that AR mRNA levels correlated significantly with α 2 mRNA levels in both benign and the Gleason 4-5 groups. These data further support the theory that AR regulates α 2 expression. Previously, Nagakawa and colleagues have reported increased levels of α 2 in the AR-stably transfected prostate cancer cell line DU-145. The AR-transfected cells also showed better adhesion to collagen I and IV than the AR-negative DU-145 cells (Nagakawa et al. 2004). The collagen fragment endostatin, which is mostly known of its decreasing effect in angiogenesis,

has recently been reported to directly interact with AR in the cytosol and inhibit transcriptional activation of AR-target genes in prostate cancer. This is believed to diminish the progression of AR-associated CRPC (Lee et al. 2015).

We also observed a significantly higher AR staining in benign samples compared to the Gleason 4-5 samples, but not a significant higher AR mRNA levels in benign specimens than in prostate carcinomas. There are controversial studies about the expression of AR during the progression of prostate cancer. Li et al. reported an increased expression of AR is associated with enhanced proliferation, aggressiveness and decreased recurrence-free survival in prostate cancer patients (Li et al. 2004). Another study showed that expression levels of AR *in vivo* do not correlate with the stages of prostate tumors (Amirghofran, Monabati, and Gholijani 2004).

6.2 Collagen binding integrins in melanoma

6.2.1 Integrin $\alpha 2\beta 1$ as a potential prognostic biomarker for advanced melanoma

According to the results in paper II, high levels of the integrin subunits $\alpha 1$, $\alpha 2$ and $\alpha 11$ have a critical impact on the survival outcome of patients with advanced melanoma. Patients with high mRNA levels of the collagen binding integrins had in general shorter survival time compared with patients expressing low levels of collagen receptors.

The most prominent result was seen with integrin $\alpha 2$. Metastatic melanoma patients with high $\alpha 2$ levels had a significantly shorter survival time in all three parameters: 1) survival after the first melanoma metastasis, 2) survival after the initiation of chemoimmunotherapy and 3) overall survival (timepoint from the primary diagnosis) compared to patients with low levels of $\alpha 2$. The statistically most significant difference was seen in overall survival. This indicates that $\alpha 2$ expression is characteristic for melanoma and that $\alpha 2$ could possibly act as a prognostic marker during melanoma treatment. Integrin $\alpha 2$ may therefore be an interesting therapy target in melanoma. These results support previous data, which indicate that integrin $\alpha 2$ is a promoter of melanoma progression, especially of the metastatic formation. High levels of $\alpha 2$ have been reported in human metastatic lesions, in xenografts models using highly metastatic melanoma cell lines, and *in vitro* in aggressive melanoma cell lines. The expression levels of $\alpha 2$ correlated with the cell adhesion to collagen type I and IV and with the cell migration in several melanoma cell lines (Klein et al. 1991; Hartstein, Grove, and Woog 1997; Schadendorf et al. 1993; Danen et al. 1993; Staniszewska et al. 2009). Integrin $\alpha 2$ is also listed as a potential biomarker of cutaneous metastatic melanoma (Vereecken et al. 2012). Our findings verify that $\alpha 2$ has potential of becoming a melanoma prognosis biomarker and can be considered as a novel therapy target in the development of anti-metastatic therapies. However, larger tissue collections are needed to confirm that $\alpha 2$ as a prognostic biomarker.

6.2.2 Integrin $\alpha 11\beta 1$ as an interesting marker for advanced melanoma

Integrin $\alpha 11$ has not been studied in melanoma before, and according to our qRT-PCR analysis integrin $\alpha 11$ is abundant in advanced melanoma *in vivo*. Expression of $\alpha 11$ was also found *in*

vitro in one metastatic melanoma cell line (SK-Mel-5), while the other investigated metastatic melanoma cell line A2058 did not express $\alpha 11$. A high expression of integrin $\alpha 11$ in advanced melanoma could be linked to shorter survival after first metastasis and after initiation of therapy. The molecular mechanism of $\alpha 11$ in advanced melanoma as well as in prostate cancer can only be speculated. As previously mentioned $\alpha 11$ might participate in the EMT process (Alcaraz et al. 2014). Integrin $\alpha 11$ is highly present in mesenchymal non-muscle cells in mouse embryos (Popova et al. 2007). Integrin $\alpha 11$ may also be active in melanoma-associated fibroblasts. As mentioned before, integrin $\alpha 11$ is strongly enhanced in cancer-associated fibroblasts in xenografts models in NSCLC. Cancer-associated fibroblasts are known to be essential stimulators of cancer growth and progression (Zhu et al. 2007; Kalluri and Zeisberg 2006). Integrin $\alpha 11$ has additionally been detected in melanoma fibroblasts, mouse dermal fibroblasts of normal origin, where it reorganizes the collagen matrix, mediates the tensile force in the matrix, and regulates the differentiation of myofibroblasts (Svendsen et al. 2009; Carracedo et al. 2010). Integrin $\alpha 11$ also controls the fluid extravasation and edema formation during inflammation in normal dermal fibroblasts (Svendsen et al. 2009). Based on the findings mentioned above, we suggest that $\alpha 11$ is an interesting target for treatment of melanoma and we encourage further studies on $\alpha 11$ in melanoma.

6.3 CT16 in melanoma

6.3.1 CT16 is a common melanoma CT antigen

Our findings demonstrated clearly that CT16 is commonly expressed in metastatic melanoma and is not present in prostate cancer. The CT16 expression in melanoma was investigated in two different collections of clinical patient samples: sera and tissues. A significant CT16 expression was detected in $\geq 60\%$ of the serum samples and in $\geq 50\%$ of the tissue samples. Our data also showed that CT16 mRNA and protein levels correlated to each other in the melanoma tissue samples.

One study has previously reported the detection of CT16 in melanoma tissues by qRT-PCR, with a medium frequency 22%. The histological status (primary or metastasis) of the melanoma tissues was not, however, described in the study (Scanlan et al. 2002a). *In silico* gene expression analyses, which screened a set of 153 CT antigens in various cancer libraries, detected high CT16 levels in melanoma (Hofmann et al. 2008). Recently, a RNA microarray study, which used clinical data from TCGA (The Cancer Genome Atlas) data portal, investigated 129 CT antigens in several types of malignancies. The study listed all CT antigens with a frequency of $\geq 15\%$ in any cancer type. CT16 was included in the list, with a high expression frequency ($\sim 40\%$) in cutaneous melanoma and with a low frequency in lung carcinoma and HER-2 positive breast cancer (Yao et al. 2014).

Even though melanoma is classified as a CT antigen rich cancer type, not many CT antigens have been reported at a protein level with a frequency around 60%. CT antigens, which have a high protein frequency in a metastatic melanoma, include MAGE-A1 (51%, (Barrow et al. 2006), MAGE-A3 (90%, (Roeder et al. 2005) and NY-ESO-1 (46%, (Velazquez et al. 2007). However, the frequency of these CT antigens is variable in different studies. The other members of the PAGE

family, which CT16 belongs to, have so far not been investigated in melanomas (CT Database). CT16 shares a 30-50 % sequence identity with GAGE antigen (Scanlan et al. 2002a). GAGE antigens which are members of the GAGE/PAGE/XAGE superfamily, have been mostly found in lung carcinoma, melanoma and hepatocellular carcinoma (CT database). GAGE antigens have however lower frequency rates (24 %) than CT16 in melanoma (Van den Eynde et al. 1995).

6.3.2 CT16 is detected in the sera of melanoma patients

Our findings showed that the optimized fluoroimmunoassay could detect CT16 very sensitively in sera of advanced melanoma patients. It did not bring out any false positive results in our healthy control group. However, for unknown reason, one sample in the commercial set of 20 healthy serum samples was positive for CT16. In general, biomarkers which are serum based are optimal and desirable, especially in follow-up analyses when evaluating the treatment responses. In addition, CT16 acted as an independent melanoma biomarker in sera when compared to the other two commonly used serum markers of melanoma; LDH and S100B. In our study, the CT16 measurement was even more sensitive in detecting melanoma cases than S100B and LDH. So far, LDH is the only FDA approved melanoma biomarker and belongs to the current American Joint Committee on Cancer staging system for cutaneous melanoma (Balch et al. 2001).

6.3.3 CT16 as a melanoma biomarker

According to the results, CT16 has a good potential to become a novel diagnosis/prognostic biomarker for advanced melanoma. CT16 is not expressed in normal melanocytes (Scanlan et al. 2002a) and based on our results it is frequently expressed in advanced melanoma. The results showed that CT16 was present both in tissues and in sera of advanced melanoma patients. However, since it is hard to find only one optimal biomarker for all conditions of the heterogeneous melanoma, many studies have suggested that a combination of various melanoma biomarkers would be the most ideal alternative. CT16 could be a novel candidate in a combination of other biomarkers. One potential combination of biomarkers could be a collection of several CT antigens. We also suggest that the measurement of CT16 could provide very valuable information when analyzing treatment responses in advanced melanoma. In our hands the measurement of CT16 levels in sera reacted more sensitively to changes in the clinical condition than S100B. However, since we only followed up the CT16 expression from four patients, broader follow-up studies with larger patient cohorts are needed to validate CT16 as a response marker for advanced melanoma treatment. In addition, further studies on CT16 expression in both primary melanomas and metastatic melanoma are required to investigate its role during the progression of melanoma.

No other expression studies on CT16 in prostate cancer have been published before, and our data indicate that CT16 cannot be classified as a prostate-associated gene, since it was not detected in prostate cancer tissues, prostate cancer cell lines or normal prostate. Two PAGE

members PAGE-1 and PAGE-4 have been associated with prostate cancer, while PAGE2, PAGE2B and PAGE-3 have not been studied in prostate cancer (CT database).

6.4 The biological role of CT16

Our findings demonstrated that CT16 can be classified as an anti-apoptotic protein. According to the microarray screen, CT16 is associated with the dysregulation of several survival/apoptosis-associated genes. This was confirmed in two separate approaches, one with CT16 knock down in melanoma cells and one with CT16 overexpression in melanoma cells. The real-time monitoring of the cell growth showed that CT16 prevented significantly the cisplatin-induced apoptosis in melanoma cells. Further, inhibition of CT16 increased the protein expression of cleaved caspase-3 in melanoma cells. One of the most interesting apoptosis-associated genes from the microarray analyses was DKK-1. CT16 was found to be a negative regulator of DKK-1. DKK-1 is generally known as an extracellular inhibitor of the Wnt-pathway, and has been found to be significantly decreased in melanoma (Kuphal et al. 2006). Many studies have shown that Wnt-signaling pathway activates apoptosis (Pecina-Slaus 2010). Overexpression of DKK-1 in melanoma cells has activated apoptosis and decreased tumor volume *in vivo* (Mikheev et al. 2007). The genes c-Myc, cyclin D1 and BIRC5 (survivin) have been found to be the most essential gene transcription targets in DKK-1 depleted breast carcinoma cells (Zhou et al. 2010). BIRC5 is known as an inhibitor of apoptosis and a cell cycle regulator. BIRC5 prevents apoptosis by binding to caspase-3 and -7 and inhibiting their activity (Tamm et al. 1998; Shin et al. 2001). Interestingly, BIRC5 was found to be significantly upregulated by CT16 in our microarray screen (not shown), but the expression difference could not be validated with qRT-PCR (IV, S4). In our paper IV, the CT16 siRNA silencing upregulated cleaved caspase-3.

Other interesting CT16 target genes, induced by overexpression of CT16, were IL-8, MT2A and FABP7 (Fatty Acid Binding Protein 7). IL-8 is a chemotactic activating cytokine induced by melanoma cells, and it correlates with the cancer progression, tumor load and the metastatic potential of melanoma cells *in vivo* and *in vitro* (Ugurel et al. 2001; Singh et al. 1994; Scheibenbogen et al. 1995). IL-8 also inhibits TRAIL- (tumor necrosis factor-related apoptosis-inducing ligand) mediated apoptosis and chemotherapy-induced apoptosis in prostate cancer *in vitro* (Wilson et al. 2008). MT2A belongs to the family of metallotheoneins, which have been suggested to be anti-apoptotic proteins with a capacity to decrease the activity of caspase-3 in neuronal cells (Helal et al. 2009). FABP7 is related to melanoma invasion and proliferation *in vitro* (Slipicevic et al. 2008; Goto et al. 2006). The dysregulation of FABP7 and MT2A was further confirmed with qRT-PCR (IV, S4).

Several CT antigens have been directly associated with cell survival. MAGE-C1 and MAGE-A3 promote the survival of myeloma cells by increasing caspase-12 and -9. They have no role in the proliferation of the myeloma cells (Atanackovic et al. 2010). GAGE decreases apoptosis through inhibition of the IFN-gamma-signaling pathway by downregulating interferon regulatory factor 1, caspase-1 and caspase-7 in HeLa- and HEK-293 cells (Kular et al. 2009). Recently, the CT antigen SSX (Synovial Sarcoma X) enhanced proliferation and survival through Wnt-pathway by upregulating c-Myc and AKT-1 (D'Arcy et al. 2014). PAGE-4, which belongs to the same family as

CT16, was identified as a member of the stress-response pathway by being a regulator of c-Jun transactivation. This action is critical for the apoptotic signaling and PAGE-4 siRNA has reduced prostate tumor growth in mouse xenografts (Mooney et al. 2014).

CT16 shares a 43 % sequence identity to PAGE-4 and both of them have been characterized as intrinsically disordered proteins (IDP) (Hellman et al. 2011; Zeng et al. 2011). Many CT-X antigens are suggested to be IDPs (Rajagopalan et al. 2011). It is common that IDP have multiple functions, compared to well-structured proteins. Some IDPs are able to transform into more stable structures by binding to their targets, like DNA. PAGE-4 has been observed to bind DNA whereas CT16 does not bind DNA (Zeng et al. 2011; Hellman et al. 2011). PAGE-4 is expressed both in normal prostate and prostate cancer (Iavarone et al. 2002) while CT16 is not present in either normal prostate or prostate cancer.

6.4.1 CT16 regulates its target genes in a p53 independent manner

Mutation of p53, which is related to many cancer conditions, does not commonly occur in melanoma (Albino et al. 1994; Sparrow et al. 1995). Various proteins may rather dysregulate the expression and function of wild type p53 through diverse mechanisms, which cause an imbalance in the p53-signaling. Some CT antigens promote cell survival and drug resistance by creating a dysregulation of p53. MAGE suppresses apoptosis through TRIM28/KRAB associated protein-1-binding and p53-degradation (Doyle et al. 2010). By interaction with histone deacetylase 2, CT antigen CAGE inhibits p53-expression and promotes drug resistance in melanoma and hepatocellular carcinoma *in vivo*. Downregulation of CAGE with siRNA led to caspase-3 activity and drug-mediated apoptosis and anchorage-independent growth (Kim et al. 2010).

According to our analyses the function of CT16 is not related to the tumor suppressor protein p53. The p53 protein stability experiment and qRT-PCR analysis in CT16 overexpressed and CT16 knock down melanoma cells resulted in no alteration of the p53-expression. There was either no effect on the conformational change on p53 (active or non-active form) in the melanoma cells overexpressing CT16. Additionally, we reported an upregulation of DKK-1 in the p53-negative Saos-2 cells. The CT antigen MAGE-A inhibits apoptosis with both p53-independency and p53-dependency by promoting the gene BIRC5 (survivin) in myeloma cells (Nardiello et al. 2011). Downregulation of MAGE-A with siRNA in p53 null cells resulted in a suppression of BIRC5 (Nardiello et al. 2011). As earlier mentioned BIRC5 is able to bind and inhibit the caspase-3 and thereby promote cell survival (Shin et al. 2001). At last, our results indicate that DNA methylation does not mediate the expression of the CT16-regulated genes. Instead the acetylation of histone H3 may be connected to the process. However, it cannot be excluded that the target genes are affected by histone H3 acetylation in a CT16 independent manner. The DKK-1 expression in colon cancer *in vitro* has been shown to be mediated by histone H3 acetylation (Wang, Li, and Chen 2012). The DKK-1 downregulation in melanoma is not regulated by DNA methylation (Kuphal et al. 2006).

7. CONCLUSIONS AND FUTURE PROSPECTS

In cancer research, it is important to find out and characterize differentially regulated genes and to understand their roles in oncogenesis, tumor progression and formation of metastasis. By identification and characterization of genes, new specific biomarkers and therapy targets may be found. In this thesis, we have characterized the collagen binding integrins and CT16 in prostate cancer, metastatic melanoma and in various cancer cell lines. In paper I, we present the first broad mRNA expression study about collagen binding integrins in 24 cancer cell lines, derived from sarcomas, carcinomas and melanomas. This report contains new *in vitro* expression data about the collagen binding integrins. Our data reveal that the majority of the cell lines express $\alpha 2\beta 1$ as their main collagen receptor, even though $\alpha 2\beta 1$ might be suppressed in the corresponding tumors *in vivo*. Integrin $\alpha 10\beta 1$ and $\alpha 11\beta 1$ were mainly present in sarcomas *in vitro*.

Based on the expression studies with the large collection of human prostate cancer specimens, it can be reported that $\alpha 2\beta 1$ is downregulated in poorly differentiated prostate carcinoma. Integrin $\alpha 2\beta 1$ was suppressed gradually in epithelial cells. This suggests that $\alpha 2\beta 1$ is important for the normal maintenance of the epithelial structure. We could also confirm, for the first time, that $\alpha 11$ expression is increased during the malignant transformation of the prostatic epithelium. These results indicate that integrin $\alpha 2\beta 1$ might partially be replaced by $\alpha 11\beta 1$ during prostate cancer progression. This alteration may reflect the EMT process (Alcaraz et al. 2014). Altered integrin expression can also cause changes in cell signaling and behavior which may impact on cell proliferation, differentiation and cell survival. Integrin $\alpha 11\beta 1$ was also detected in the stromal part, which may indicate that it participates in tumor-stromal interactions, likewise its role in lung cancer (Zhu et al. 2007; Navab et al. 2015). Additionally, we could verify the association between AR- and integrin $\alpha 2\beta 1$ - expression in prostate cancer lesions, which has been detected before *in vitro*. We encourage further research on $\alpha 2$ and $\alpha 11$ in prostatic carcinoma, especially about the suppression of $\alpha 2$ and the potential role of $\alpha 11$ in epithelial cells and stroma. In the nearest future it would be interesting to study further the expression of $\alpha 11$ and $\alpha 2$ in specific subpopulations of cells, e.g. cancer stem cells and cancer associated fibroblasts. Specific markers could be used in the immunostaining of the samples. Similar analysis using samples of prostate cancer metastases could bring further information. Profiling the changes in gene expression could be used to investigate the putative differences in signaling by $\alpha 2$ and $\alpha 11$.

In paper II, in which we studied the impact of collagen binding integrins on progression of metastatic melanoma, the mRNA levels of $\alpha 1$, $\alpha 2$ and $\alpha 11$ were significantly associated with survival. High levels of integrin $\alpha 1$, $\alpha 2$ and $\alpha 11$ increased the risk of short survival. Especially, integrin $\alpha 2\beta 1$ has the potential to be a novel indicator for poor prognosis, since it was significantly associated with shorter overall survival time. However, larger tissue collections are needed for further conclusions. It would also be of interest to investigate the expression of collagen binding integrins in primary melanomas. Especially since there is a highly need of biomarkers in the early stage of melanoma. Additionally, the expression of collagen binding integrins could be followed up during the new melanoma treatments using BRAF- and CTLA-4 inhibitors, since they may act as specific biomarkers for efficacy. Our observations also suggest

that $\alpha 2$ is a potential target for future drug development. Therefore, its biological role during the process of malignant progression should be further studied.

In this thesis we reveal new information about CT16. We report new data about its expression in cancer and we announce the first biological study about the role of CT16 in melanoma. The results evidently demonstrated that CT16 is not expressed in prostatic carcinoma but is frequently expressed in metastatic melanoma. CT16 can be measured in very sensitive manner in sera using a novel fluoroimmunoassay. The sensitivity is a very critical factor for the validations of biomarkers. Likewise, the measurability in sera is a highly prioritized factor when evaluating new biomarkers. Furthermore, the amount of CT16 seemed to reflect the alterations in the clinical condition of four patients followed in the preliminary study. To conclude, these results mean that CT16 has potential to be a sensitive diagnosis marker and a good prognostic/treatment response marker for metastatic melanoma. However, larger collections of follow-up samples are required to be able to draw any further conclusions. Measurement of CT16 concentration in sera of primary melanoma patients would also be valuable since it is critical to detect melanoma in its early stage.

It would also be interesting to analyse the serum levels of CT16 in renal cell carcinoma since CT16 is expressed with a relatively high frequency (44 %) in the disease (Scanlan, Simpson, and Old 2004). Renal cancer has usually a low frequency of CT antigens. In a study by Scanlan et al. only six out of 25 CT antigens had an expression frequency over 10 % in renal cancer (Scanlan, Simpson, and Old 2004).

According to our results we can report that CT16 is an anti-apoptotic gene in a p53 independent manner. CT16 regulated the expression of several apoptosis-related genes such as DKK-1. CT16 inhibited the activation of caspase-3 and was able to protect metastatic melanoma cells against cisplatin-induced cell death. However, the precise CT16-survival mechanism remains to be found. According to our data, Wnt-pathway seems to be involved since DKK-1, the inhibitor of Wnt-signaling pathway, was significantly downregulated by CT16. DKK-1 induces apoptosis in brain tumor, melanoma and interestingly also in renal cell carcinoma, which also has a high frequency of CT16 (Shou et al. 2002; Hirata et al. 2011; Mikheev et al. 2007). According to our results, CT16 is a putative drug target for promoting cell death in the metastatic melanoma cells.

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